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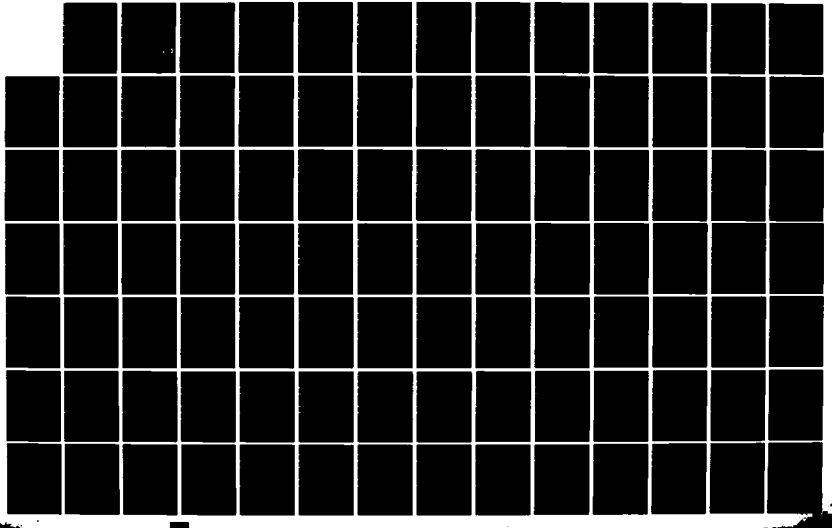

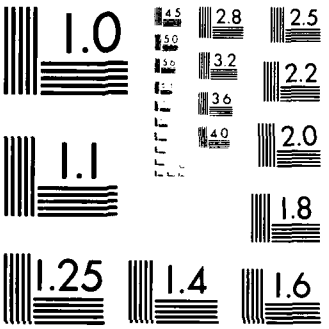

# CHEMICAL-INDUCED ERYTHROCYTOSIS IN WISTAR RATS: ASSESSMENT AS A MODEL FOR HUMAN POYLCYTHEMIA

By

GERRY MICHAEL HENNINGSEN

A dissertation submitted in partial fulfillment of
the requirements for the degree of

DOCTOR OF PHILOSOPHY

WASHINGTON STATE UNIVERSITY
Pharmacology/Toxicology Program

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# CHEMICAL-INDUCED ERYTHROCYTOSIS IN WISTAR RATS: ASSESSMENT AS A MODEL FOR HUMAN POLYCYTHEMIA

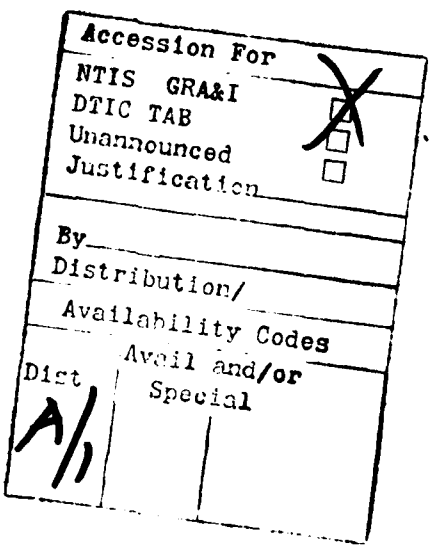
Abstract

by Gerry Michael Henningsen, DVM, PhD
Washington State University
May 1985

Chair: Loren D. Koller

Polycythemia is a hematological disease characterized by erythrocytosis and occasionally leukocytosis. Many types of polycythemia occur in man and animals due to hemoconcentration, hypoxia, inappropriate production of erythropoietin, or more rarely, autonomous production of blood cells. The purpose of this study was to reproduce and characterize a polycythemic condition produced in a closed colony of Wistar rats. Following transplacental exposure to methylmercury, ethylurea, and sodium nitrite, the rats developed up to a 41% incidence of polycythemia. Clinical signs of polycythemia developed in offspring from all methylmercury-treated groups, with the highest incidence seen in groups exposed to all three chemicals. Most cases appeared at 8 weeks of age with fewer cases between 3 and 9 months of age. Chronic methylmercury treatment of the dams was essential, and butylnitrosourea plus methylmercury was also capable of inducing the disease. Hematocrits in afflicted animals ranged from 60 to 84%. Blood cell morphology and differential counts were generally normal. The red cell mass was significantly elevated which was indicative of absolute erythrocytosis. Marrow cell culture studies and elevated erythropoietin levels in diseased rats were diagnostic of secondary poly-

cythemia; however, no apparent secondary causes were detected. Erythrocytes from polycythemic rats were previously found to have an increased affinity for oxygen, but no hemoglobin abnormalities were initially detected. Oxygen affinity of hemolysates remain to be tested. Splenomegaly, marrow hyperplasia and variable leukocytosis and thrombocytosis present in this secondary polycythemic condition are unusual features that are more typically found in polycythemia vera, an autonomous myeloproliferative disorder in man that results from clonal neoplasia of bone marrow stem cells. However, the data described in these investigations revealed the polycythemia is secondary and reproducible in a closed colony of Wistar rats exposed to transplacental chemicals. Presently, this disease offers considerable potential for furthering the knowledge of hematopoiesis and may eventually serve as a model for secondary polycythemia in humans.



To the Faculty of Washington State University:

The members of the Committee appointed to examine the dissertation of GERRY MICHAEL HENNINGSEN find it satisfactory and recommend that it be accepted.

Chair

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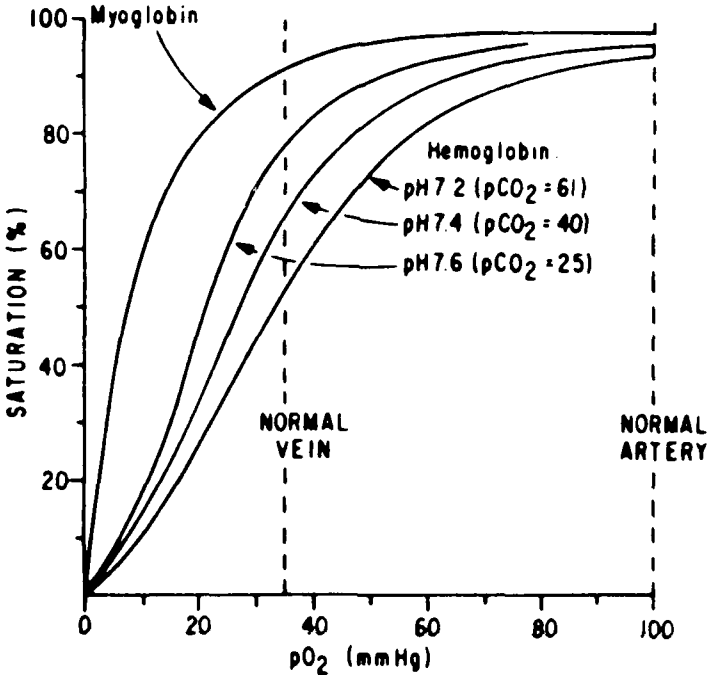


Figure 2. Oxygen - Hemoglobin Dissociation Curve.

A normal $O_2$-Hb curve is sigmoidal and reflects a large release of $O_2$ from Hb over a narrow $PO_2$ range. High affinity for $O_2$ causes a left shift in the curve which becomes more hyperbolic. Low affinity for $O_2$ causes a right shift in the curve and more $O_2$ release at higher $PO_2$ levels. The $P_{50}$ value is the $PO_2$ in mm Hg where 50% of the Hb is saturated with $O_2$. The $P_{50}$ value indicates affinity for $O_2$. (Reproduced from pg 94, Wintrobe, 1981, ref. no. 175).

absolute erythrocytosis [4,17]. Plasma and blood volume measurements are more useful for monitoring relative erythrocytosis, shock or hemorrhage [175].

Oxygen saturation. Arterial $O_2$ saturation ($S_aO_2$) measurements indicate how efficiently $O_2$ is being loaded into the blood. Normally $O_2$ saturation is greater than 92% of maximum for human blood and 89% for rat blood [17,145]. One gram of hemoglobin binds 1.34 ml $O_2$ when fully saturated [175]. Normal saturation of arterial blood ($S_aO_2$) at sea level is 97% for humans and 91% for rats which is equivalent to a $P_aO_2$ of 97 and 95 mm Hg, and $P_{50}$ of 26.5 and 38 mm Hg, respectively [174]. The $P_{50}$ value is the $O_2$ tension of blood when 50% of the Hb is oxygenated. This value estimates $O_2$ affinity of the blood or hemoglobin and is calculated from an oxygen-dissociation curve (Figure 2) [175]. Systemic hypoxia results when $O_2$ saturation drops below 92%, and homeostatic mechanisms act by stimulating erythropoiesis to increase oxygen transport [68].

Arterial $O_2$ saturation by itself does not always provide an accurate picture of tissue oxygen delivery [65, 175], since up to ten biological factors can influence the release of oxygen to tissues. Also, problems with $O_2$ unloading from Hb or delivery of $O_2$ to renal oxygen sensors can produce secondary-appropriate erythrocytosis when $O_2$ saturation is >92% [4]. Cases of polycythemia with concurrent low $O_2$ saturation and PV (11% may have $S_aO_2$ <92%) or high-affinity hemoglobinopathies complicate diagnosis [17,175].

Blood cell counts. Erythrocytes and leukocytes may be counted manually with a hemocytometer or automatically for more precision and convenience [145,175]. Red cell indices may also be calculated to provide values for MCV (mean corpuscular volume), MCH (mean corpuscular hemoglobin), and MCHC (mean corpuscular hemoglobin concentration). These indicies rarely help to diagnose erythrocytosis [5].

Erythrocyte morphology is generally normal, and nucleated RBCs or reticulocytes are seldom encountered in polycythemia [55,175]. Although some hematologists place considerable diagnostic importance on marrow examinations for erythrocytosis, most agree such exams are of little value [4,55,70,90,175]. Most primary erythrocytosis patients have hypercellular marrow and megakarocyte hyperplasia, two features which may assist in the diagnosis of PV [44,55].

Red cell mass and volume. Red cell mass measurements are made with a technique that uses $^{51}Cr$-labeled RBCs and measures their dilution in the blood stream. Units are reported as ml blood /kg body weights . Total blood volume can be calculated with the Hct value, or more accurately determined by adding the plasma volume (determined with Evans Blue dye or $^{131}I$-labeled serum albumin) to red cell mass. As simply a measure of erythrocytosis, the Hct is superior to red cell mass due to less variation in the population's Hct [175]. As a result, the Hct may detect an early case of polycythemia before the red cell mass becomes significantly elevated above population norms. Red cell mass is valuable, however, in differentiating relative from

92%, and splenomegaly, (three major criteria); or b) the first two major criteria (excluding splenomegaly) plus any two of the minor criteria: thrombocytosis, leukocytosis, increased leukocyte alkaline phosphatase, or increased serum $B_{12}$ [17].

Applicable diagnostic tests for polycythemia are outlined below:

Hematocrit. The packed cell volume (PCV), or hematocrit (Hct), is often the first laboratory test performed to test for erythrocytosis [4,17,55,129,175]. The Hct expresses the percentage of blood sample occupied by erythrocytes. Elevated hemoglobin (Hb) concentrations often correspond with the elevated Hct [4,55]. Erythrocytosis is diagnosed (depending on the physician) when the Hct exceeds 50-54% in males and 47 - 50% in females, or when Hb exceeds 17-18% in males and 16-17% in females [4,55,129].

The PV Study Group showed that absolute erythrocytosis was present in only 15% of patients with a Hct of 50 to 51%, 40% with a Hct of 55%, and over 90% of patients with a Hct $\geq$ 60% [17]. Females presumably have lower Hct and Hb values due to menstrual blood loss, estrogen effects, and lack of androgens [4,175].

Hemoglobin concentration. Cyanmethemoglobin is the standard form of hemoglobin to be assayed for concentration in blood [175]. Precise amounts of blood and cyanide reagents are mixed to form hemoglobin-cyanide. Absorbence of light at 540 nm is measured on a spectrophotometer and compared to standards. Hemoglobin concentration is expressed as g/dl of whole blood.

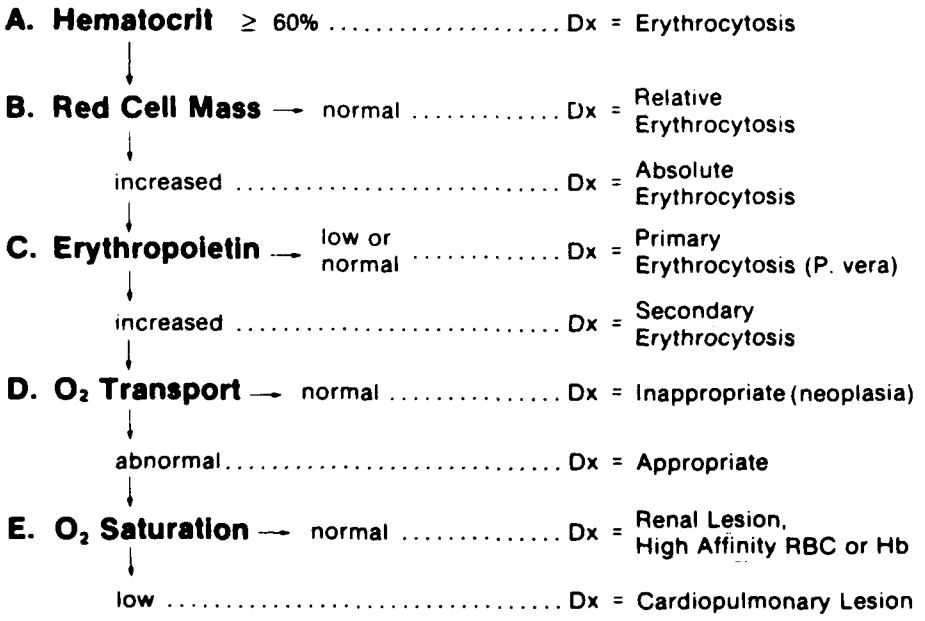


Figure 1. Diagnostic Algorithm for Polycythemia.

Polycythemia, or erythrocytosis, was diagnosed in rats whose hematocrits exceeded 60%. Red cell mass and erythropoietin levels aid in the classification of polycythemia, and oxygen transport studies help determine secondary causes of polycythemia. (Algorithm suggested by Adamson, 1983 and 1984, ref. nos. 4,5).

depending upon the role of Ep. Secondary erythrocytosis is Ep-dependent, and elevated levels of Ep can usually be detected in the plasma, serum or urine [4,115]. In contrast, primary erythrocytosis is Ep-independent. A composite of several recent classification schemes is shown in Table 1 to represent the current paradigm for polycythemia [5].

d. Diagnosis

Diagnostic algorithms are published which allow practitioners a methodological approach towards determining the class and type of polycythemia under study (Figure 1) [4,17,55]. It is critical to correctly diagnose polycythemia so as to implement the indicated therapy or avoid a contraindicated treatment [4,175]. Most hematologists subjectively consider relative polycythemia to be the most common form [17].

Even with refined laboratory techniques and better understanding of the polycythemias, diagnostic classification of polycythemia may be more complex than previosly supposed [115]. One reason for this difficulty is the occasional unexplained case that is usually due to recessive familial abnormalities [1,2,4,22,32,60,179]. Diagnosis of PV continues to be one of the most difficult. As a result, the Polycythemia Vera Study Group established a set of criteria for the diagnosis of PV [17]. Patients must 1) not have received treatment except for phlebotomy, 2) have been diagnosed as polycythemic less than 4 years ago, and 3) fulfill either the following diagnostic categories: a) elevated red cell volume, arterial $O_2$ saturation $\geq$

first adequately described PV in 1903, and the first familial occurrence of PV was recognized in 1907 by Nichamin [45].

As early as 1906, Carnot [45] proposed a humoral factor to mediate an increase in circulating RBCs as a result of hypoxia, but it was not until 1950 that this factor, erythropoietin (Ep), was demonstrated to exist by experimental evidence [140]. Erythropoietin is a renal hormone that adjusts the size of the red cell mass (RCM) to the need for oxygenation of tissues. Erslev, in 1953 [45], was the first to clearly identify Ep. A technique to obtain purified Ep was recently developed in 1977 by Miyake et al. [108], and this led to the recent development of long-awaited, sensitive, immunoassays with which to study Ep and eythropoiesis [52].

Despite the long awareness and recent scientific advances by researchers, polycythemia has remained an enigma to the biomedical profession --especially PV [12]. The successful use of the Lyon hypothesis by Adamson, et al, demonstrated that polycythemia vera is a neoplasia of a clonal pluripotent stem cell [3]. Researchers continue to study PV, hemoglobinopathies, thallasemias, and a multitude of other hematological disorders with a major goal of unraveling the intricate mechanisms of hematopoiesis [45,118,175].

c. Classification

Erythrocytosis can involve either a relative or an absolute increase in RBC concentration. Absolute erythrocytosis is differentiated by an increase in red cell mass and blood volume [175]. Absolute erythrocytosis is classified as either primary or secondary

and stroma cell lines [55]. These diseases are commonly termed 'myeloproliferative disorders', or better, 'myelodysplastic disease' which emphasizes both the qualitative and quantitative abnormalities of stem cell growth [55]. Erythroleukemia is another example of a myeloproliferative disease which is characterized by malignant growth of red, and sometimes white, blood cells. Erythroleukemia differs from PV by producing severe anemia and abnormal circulating erythrocytes.

b. Background

Polycythemia occurs with an incidence of about 4% in the human population [68]. The majority of cases develop in response to some form of hypoxia [68,175]. Polycythemia only rarely develops from autonomous erythropoiesis or exogenous erythropoietin production. The incidence of PV ranges from about 0.5 to 1.6 per 100,000 United States residents [12,21,55] and is slightly higher for members of the Jewish race, Caucasions vs blacks, and men [93,175]. Most familial polycythemias involve mutant hemoglobins with high affinity for oxygen, but familial occurrence has been observed in some cases of PV and other cases of absolute erythrocytosis [21,138,175].

Some of the first observations of clinical erythrocytosis were made by the French physician, Dr. Dennis Jourdanet, who noted symptoms of hypoxemia and observed increased viscosity of blood. In 1890, Viault first showed that erythrocytosis develops as an acclimation to high altitude exposure. Vaquez is credited with originally describing persistent polycythemia in 1892 based on elevated RBC counts. Osler

# II. LITERATURE REVIEW

## A. POLYCYTHEMIA

### 1. Overview

#### a. Definitions

'Polycythemia' is a descriptive term that indicates an elevation in hemoglobin (Hb) concentration and erythrocyte packed cell volume (PCV, hematocrit) of the peripheral blood [55,65]. The Hb and PCV measurements only express a ratio between erythrocytes and plasma, and do not provide information on the absolute concentration of RBCs in the intravascular space. Thus, 'polycythemia' is no longer considered an acceptable stand-alone diagnostic term [55]. The appropriate modifying adjective for the observed 'polycythemia' should be used to describe the nature of the altered ratio of RBC to plasma concentrations.

Many hematologists prefer the term 'erythrocytosis' when describing an increased concentration of erythrocytes [5,132,175]. Polycythemia literally means "many cells", and is generally used to denote an increase in RBCs while disregarding numbers of leukocytes and platelets [175]. 'Erythremia' implies that leukocytes and platelets are also absolutely increased in numbers, and this type of hematological disorder is known as polycythemia vera (PV). Neoplasia of a clonal pluripotent stem cell produces PV [3]. Polycythemia vera is considered to be one of a group of closely related diseases characterized by disordered proliferation of precursor hematopoietic

polycythemia vera, would prove most useful. However, this rat disease could potentially provide a useful tool in the form of a chemical-induced secondary polycythemia with which to study the mechanisms of erythropoiesis. To properly diagnose the rat polycythemia and determine its etiology, we used an algorithm suggested by Dr. John W. Adamson [5] (Figure 1). Packed cell volumes, or hematocrits (Hct), were measured weekly, and erythrocytosis was diagnosed when the Hct exceeded 60%. The next steps were to determine red blood cell mass and, if elevated, determine Ep levels. Additional clinical and biological tests would be conducted as indicated by the algorithm in order to adequately characterize the polycythemia. Varied exposure regimens and chemical analogs were also examined for their effects on the production of polycythemia. Parental lineage was monitored to observe genetic influence on phenotypes, and treatments of mice and other rat strains were performed to confirm the unique susceptibility of this particular Wistar colony to the transplacental chemicals. This dissertation provides background and findings for each of the aims used to describe the secondary polycythemia finally diagnosed in these laboratory rats.

exposed to MM, EU, and $NO_2$ [81]. Complete characterization of the disease was impractical then, and subsequent attempts to reproduce the chemical-induced polycythemia in other species and strains of rats failed. Investigators thus suspected that this closed colony of Wistar rats was genetically vulnerable to chemically induced polycythemia. An intriguing aspect of this disease was its similarity to human polycythemia vera - a neoplastic disease of clonal pluripotent stem cells in the bone marrow [3]. The similarities included elevated blood counts, hemoglobin levels, and packed cell volumes, as well as splenomegaly, hyperplastic bone marrow, and megakaryocytosis. In addition, hydration, hemoglobin analysis and 2,3-diphosphoglycerate (2,3-DPG) levels were all normal in polycythemic rats; however, an unexplained increase in oxygen affinity of whole erythrocytes was found in polycythemic rats. No secondary causes of polycythemia were detected in the heart, lungs, kidneys, or other organs through gross and microscopic pathological examinations. The prospect that this disease could provide an animal model for studying primary polycythemia in humans was exciting since an adequate model did not exist [132].

The aims of this research were a) to attempt to reproduce the polycythemia in rats from the original Wistar colony, b) to diagnose and characterize the disease as completely as possible, c) to investigate the pathogenesis and hopefully determine the target tissue affected by the toxicants, and d) to assess the disease as a model for human polycythemia. A model for the human neoplastic disease,

# INTRODUCTION

Polycythemia is a hematological disease characterized by an increase in concentration of blood cells. Red blood cell concentration is always elevated and is termed 'erythrocytosis', while white blood cells and platelets are variably increased [175]. Polycythemia may be due to either, or both, a physiological or pathological disorder which affects hematopoiesis. Because polycythemia can arise from a myriad of unrelated causes, proper classification assists in the diagnosis of polycythemia and in determining its etiology [4,17]. Absolute polycythemia is diagnosed by an increased red cell mass. Within this category, secondary polycythemia patients manifest elevated erythropoietin (Ep) levels in contrast to normal or low levels in primary polycythemia patients. The kidney produces the hormone, Ep, as a normal homeostatic response to hypoxia [175]. Erythropoietin principally stimulates erythroid progenitors known as colony-forming units (CFU-E) which proliferate to increase the red cell mass and oxygen delivery to tissue.

This study involved chemical-induced polycythemia that developed in Wistar rats from a closed breeding colony originated at Oregon State University in 1934 [81]. In the late 1970's, investigators used these rats to examine the cocarcinogenic effects of methylmercury (MM) on the development of neurogenic tumors induced by transplacental exposure to ethylnitrosourea (ENU) administered as the precursors, ethylurea (EU) and sodium nitrite ($NO_2$) [120]. Polycythemia was an incidental finding that developed in up to 41% of the offspring

# LIST OF FIGURES

Erythropoietin levels. Analysis of Ep levels in the serum, plasma, or urine is used to differentiate primary from secondary erythrocytosis [4,17,20,115]. The rather crude, insensitive, and laborious mouse bioassay (exhypoxic or hypertransfused) was the standard Ep assay for years which quantified Ep by measuring $^{59}Fe$ incorporation in circulating RBCs [47,175]. Recently, Ep has been purified and cloned, and a radioimmunoassay and other sensitive Ep assays have been developed which can detect <1mU Ep/ml serum [47,52, 89,108]. The mouse bioassay only had a lower detection limit of about 30 to 50 mU Ep/ml blood [132], but lately it has been modified to detect levels of Ep down to 5 mU/ml [154]. Normal human plasma levels are about 5 to 20 mU Ep/ml [115].

In one study using the RIA, 94% of secondary polycythemia patients had Ep levels >30 mU/ml whereas 92% of PV patients had Ep levels <30 mU/ml [115]. Several documented cases of primary erythrocytosis with elevated Ep levels are perplexing [2,39,115,171]. Classic PV patients feature Ep-independent erythropoiesis in vitro [56], yet cases of apparent PV have occasionally displayed Ep-dependent erythropoiesis in culture [42,60,91]. Autonomous erythropoiesis in culture may be indicitive, but not pathognomonic for PV [150].

Cases of erythrocytosis having normal $O_2$ saturation levels and elevated Ep levels without local or systemic hypoxia are classified as secondary-inappropriate erythrocytosis. These patients have autonomous Ep production exceeding amounts needed for adequate tissue

oxygenation [4]. Most cases involve exogenous Ep secretion by neoplasms, of which over half originate in the kidney [20]. Recessive familial production of excess Ep also accounts for some cases [4,60]. In the above cases as well as in PV patients, phlebotomy does not usually cause a subsequent elevation of Ep secretion [2,88,115]. Secondary-inappropriate erythrocytosis differs from the class which arises from local renal hypoxia caused by renal vascular disorders, renal cysts, hydronephrosis and renal transplants [4,17,20,68]. These cases of erythrocytosis are thought to result from an appropriate increase in Ep production by the kidney in response to local ischemic lesions, even though $S_aO_2$ levels are normal [20,175]. Phlebotomy often results in a dramatic increase in Ep levels which help differentiate these cases of polycythemia [4,88].

Diagnosis in animals. A similar diagnostic algorithim can be used in animals as is employed with human polycythemia. Most classes of polycythemia in humans have also been recognized in animals, principally dogs [145]. Excitable horses and dogs can exhibit transient relative erythrocytosis from splenic contraction, a condition not recognized in humans [145,175]. Also, Hcts vary with breeds such as in the greyhound where the values are commonly 60% [40]. Spontaneous polycythemia has been reported in dogs, cattle, cats, horses, rabbits, rats, and mice [13,69,106,113,116,132,159,167,173]. Experimental polycythemia has been produced in hypertransfused or exhypoxic mice [175], in rats given intrarenal $Ni_3S_2$ [72], in Wistar rats exposed transplacentally to methylmercury and ethylnitrosourea

[81], in rats injected with cobalt [175], in newts exposed to lactate [49], and in hyper-transfused dogs [65].

Polycythemia vera has been reported in dogs, cattle, a cat and possibly a horse and rats; however, notable exceptions in the animal and human forms of PV exist [13,81,132]. Splenomegaly, leukocytosis, and thrombocytosis frequently occur in human PV patients but are rarely seen in animals [132]. Most PV reports in animals are more accurately classified as primary erythrocytosis since only RBCs are usually elevated [5,7,132].

Hemoglobinopathies result from changes in the normal amino acid sequence of globin, which usually involves the substitution of a single amino acid [152]. More than 60 structural variants of Hb with increased $O_2$ affinity have been identified since Charache reported the first case, Hb Chesapeake, in 1965 [28]. Few spontaneous cases have been observed in animals [8,147], although mutant Hbs have been induced in irradiated mice [149,173]. No cases of erythrocytosis have been diagnosed in association with the animal cases of hemoglobinopathies [77,149].

Diagnosis of primary polycythemia in animals is based on an elevated Hct, documenting absolute polycythemia with red cell mass determinations, excluding secondary causes of absolute polycythemia, and failure to detect elevated circulating Ep [40,131,132]. With the advent of available and sensitive Ep assays, Dr. J.W. Adamson suggests a more direct diagnostic algorithm for polycythemic animals as shown in Figure 1 [5].

e. Clinical Features

The clinical manifestations of erythrocytosis arise from two principle sources [175], the first being the underlying disorder, e.g., congenital heart disease or chronic pulmonary obstruction. The second source is the increased blood volume and increased blood viscosity which by themselves produce certain signs and symptoms [132,175]. These ailments worsen as the degree of erythrocytosis becomes more severe. Headache, weakness, dizziness, tinnitus, paresthesia, dyspnea, epigastric pain, joint pain, thrombosis, and plethora or cyanosis can occur in all polycythemias (especially the absolute type) regardless of cause [65,93,169,175].

2. Erythropoiesis

a. Erythron

Only a century ago did man realize that blood cells originate from the bone marrow. In 1929, Boycott conceived the useful idea that erythrocytes and their precursors make up a single organ termed the 'erythron'. Its interstitial tissue consists of plasma plus the fat and reticulum of the bone marrow, making the erythron a larger organ than the liver [175].

Ontology. The bone marrow produces most of the peripheral blood cells except for lymphocytes [58,175]. In mammalian embryos, blood cell production begins in the yolk sac and later proceeds to the liver and spleen in the fetus. Embryonic hemoglobin (Hb) disappears and fetal Hb takes over, except for dogs, cats and horses which do not develop fetal Hb [145]. By parturition the fetal bone marrow produces

nearly all blood cells [175]. The spleen, however, retains hematopoietic capacity through adult life [145]. Bone marrow cells develop extravascularly within a network of reticulum. Erythrocytes develop in small clusters around a central macrophage known as a nurse cell which assists in erythropoiesis [175]. A hematopoietic inductive microenvironment (HIM) provides favorable intramedullary factors for hematopoiesis [65,175]. The principle sites of erythropoietic activity vary greatly with age and species [163].

b. Erythroid progenitor cells

Erythroid cell differentiation represents one of nature's most profound examples of biologic specialization [118,175]. Within 7 to 10 days, an undifferentiated stem cell gives rise to 16 or 32 mature erythrocytes that consist of little more than a membrane surrounding a solution of protein and electrolytes [40,175]. Over 95% of the protein is hemoglobin which transports oxygen [118]. The mature RBC lacks a nucleus, mitochondria and ribosomes. It represents the culmination of complex cellular and molecular events by which the primitive stem cell ultimately becomes committed to the highly selective expression of a few genes [118].

Most information on hematopoietic stem cells has been obtained from murine studies. Evidence supports the existence of both pluripotent and unipotent stem cells [175]. A schematic view of the cellular stages of murine erythropoiesis and their regulation is provided in Figure 3 [109]. A similar model is proposed for human hematopoiesis [175].

Basically, a totipotent hematopoietic stem cell (HSC) is thought to give rise to lymphoid and myeloid stem cells (CFU-S). The pluripotent myeloid stem cell produces unipotent stem cells of the granulocyte, monocyte, megakaryocyte and erythrocyte (BFU-E) series. The macro-BFU-E gives rise to two, more mature burst-forming unit-erythroid (BFU-E) progenitors. As these committed stem cells differentiate, they lose burst-promoting activity (BPA) sensitivity and proliferative capacity. The BFU-E differentiates into the colony-forming unit-erythroid (CFU-E) which is Ep-dependent and BPA-independent [42]. The CFU-E gives rise to the cluster-forming cell which in turn produces the first visably recognized erythroid progenitor -- the proerythroblast, or rubriblast in veterinary nomenclature [40].

d. Immune control

Research is currently expanding to define the exact role immunity plays in blood formation, either directly through helper or suppressor activity at the progenitor level, or indirectly by increased demand placed on precursors through depletion of mature circulating elements. Malfunction of the immune system could possibly underlie many hematological disorders, including all forms of myelogenous malignancy [15,58].

Prostaglandins (PG) A, $E_1$, $E_2$ and $F_2$ have all shown erythropoietic stimulatory activity [100]. Lymphocytes and macrophages are presumably the sources of various colony-stimulating factors (CSF) and burst-enhancing factors or burst-enhancing factors or burst-promoting

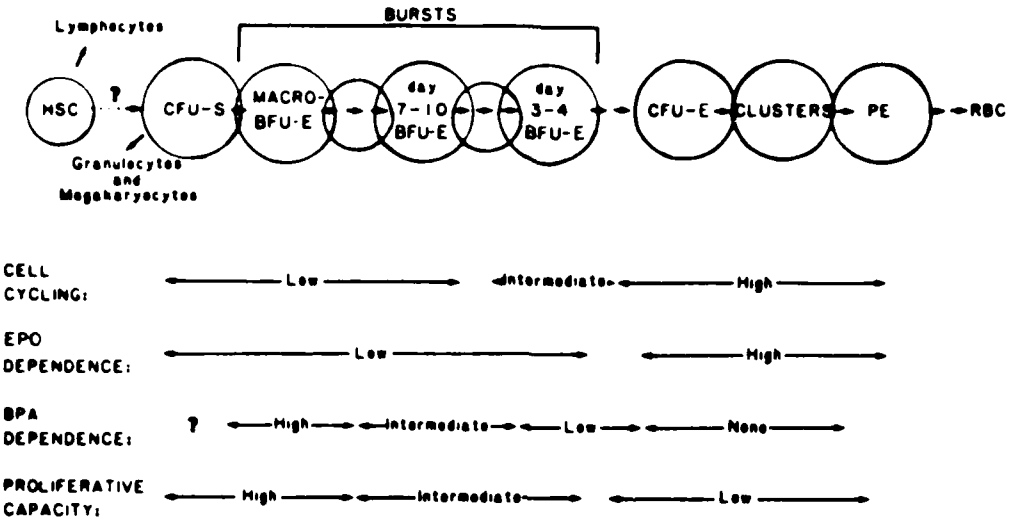


Figure 3. In Vitro Erythroid Progenitors from Bone Marrow Cells.

The erythrocyte (RBC) precursors mature form left to right. The totipotent stem cell (HSC) gives rise to a pluripotent stem cell (CFU-S) which in turn produces a unipotent erythroid stem cell (Macro BFU-E). Three stages of maturity have been identified for the BURSTS which eventually produce erythroid colony forming units (CFU-E). The CFU-E form clusters of erythroid precursors that develop into pro-erythroblasts and eventually RBCs. These progenitors vary in mitotic activity and in erythropoietin (EPO) and burst-promoting activity (BPA) dependence according to maturity. (Reproduced from page 39, Monette, 1983, ref no. 109).

activity (BPA), as well as PG and interferon [42, 59]. Four types of CSFs have been purified which function as glycoprotein growth factors and arise from many body tissues that contain activated lymphocytes, fibroblasts or endothelial cells. The multi-CSF appears to be analogous to BPA, interleukin 3, erythroid-CSF, and other factors [117].

Although definite, repeatable effects on erythropoiesis by the immune system are seen in laboratory animal and in vitro cellular experiments, no clear role has emerged [58]. Evidence suggests or infers such an immune role, but conflicting data and disagreement on the relevance of cell cultures or selected animal studies do not yet permit any strong conclusions relating hematopoiesis and immunity.

d. Erythropoietin

Erythropoietin (Ep) is a single chain, acidic glycoprotein with an apparent molecular weight of 39,000 and a carbohydrate content of about 10%. The carbohydrate, sialic acid, prolongs Ep's in vivo activity [175]. Purified Ep has a biological acitivity of 70,000 units per mg protein or roughly one unit per 14 pg or 0.35 picomole [108]. The previous unit of biological acitivity was defined as the erythropoietic effect of 5μM $CoCl_2$ in a fasted rat [45].

The biological half-life of Ep in rodents is about 90 min, and less than 24 hrs in humans [45]. Erythropoietin is generally not species specific [145]. It is slowly excreted in the urine at 1 to 4 units per liter (about 1/3 of the plasma concentration), and is also

thought to be degraded by the liver and kidney [175]. The concentration of Ep in plasma is generally proportional to the Hct and level of hypoxia [45].

The major site of Ep production in adult mammals is the kidney; this is the exclusive site of production in the dog [45,145,175]. The liver (Kupffer cells) appears to be the major site of Ep production during fetal and neonatal development [145,175]. The liver also produces 10 or 15% of the total Ep in adult rat serum, but this extra-renal Ep may be more labile than the renal form [45]. Maternal Ep does not cross the placenta to any great extent. Neonatal rats switch from extra-renal to renal Ep production between 1 and 4 weeks of age [45,145]. A postulated Ep precursor, erythrogenin, probably does not exist [6,45,175].

Cellular hypoxia initiates appropriate Ep production, but the exact renal cells responsible for translating oxygen pressure into hormone synthesis are still unknown [4,45,50]. Dr. J.W. Adamson argues that the venous or postarterial limb of the vascular tree in the kidney is the physiological-predicted site for Ep production [4]. This site would account for the cases of hemoglobinopathies where arterial $O_2$ saturation is normal, but venous $O_2$ tension is abnormally low and Ep levels are thus elevated in response to hypoxia [2,48]. However, two main bodies of evidence presently support either the juxtaglomerular apparatus (JGA) or epithelial cells of the glomerulus as the cellular site of Ep origin [48,175].

As with other polypeptide hormones, Ep presumably acts via surface receptors and intracellular secondary messengers. Early effects are seen as increased transcription followed by the appearance of mRNA in erythroid progenitors [45,175]. Erythropoietin stimulates both maturation and proliferation of erythroid stem cells, causing marrow hyperplasia and increased red cell mass [175]. Studies also suggest Ep can affect more mature RBCs by shortening or skipping mitoses, speeding up denucleation, and releasing reticulocytes earlier into the circulation [45,175].

e. Erythrocytes

The erythrocyte (RBC) is designed to transport oxygen via the protein hemoglobin. The disc shape of an RBC provides a nearly maximum surface-to-volume ratio to facilitate gas transfer, and an elasticity to enhance microvascular flow through capillaries as small as 7 mm diameter [175]. Erythrocytes average 7.8 mm in diameter and 90 fl volume in humans, and about 6.3 mm and 60 fl MCV in rats [145,175]. Both the diameters and MCV decrease with the age of most mammals [145]. The erythrocyte number increases with age so that in normal adults it averages about $5 \times 10^{12}/l$ in man or $8 \times 10^{12}/l$ in rats [145,175]. Females have slightly fewer RBCs but larger MCVs to compensate for equivalent oxygenation. Average life span of human erythrocytes is 120 days compared to 55 days for rats [145]. Erythrocytes are destroyed primarily through extravascular hemolysis which takes place in splenic macrophages [175].

(1) Metabolism

Erythrocytes have no mitochondria for oxidative metabolism and must rely on anaerobic glycolysis for energy [68]. Four major metabolic pathways maintain RBC and Hb integrity and function [175]. The glycolytic (Embden-Meyerhof) pathway provides 1 or 2 moles of ATP per mole of glucose consumed. The pentose phosphate pathway is an ancillary energy system that couples oxidative metabolism with reduction of NADP and glutathione. The NADH methemoglobin reductase pathway protects the heme iron from oxidation. The fourth metabolic pathway is the Luebering-Rapaport shunt which allows 2,3-DPG to accumulate to about 1 mole per 1 mole Hb [68]. Levels of 2,3-DPG regulate Hb affinity for $O_2$ as well as $O_2$ transport. With increased tissue hypoxia more 2,3-DPG binds Hb and thereby glycolysis is increased to produce more ATP and 2,3-DPG. The elevated 2,3-DPG, along with acidosis (Bohr effect), causes a right shift in the $O_2$ dissociation curve which increases $O_2$ delivery to the tissues [68].

Metabolic disturbances in the above pathways (enzymopathies) can disrupt normal RBC function so that $O_2$ transport is impaired [164]. Secondary polycythemia could conceivably occur in patients with deficient glycolysis, severe methemoglobinemias, denatured Hb from glutathione deficiency, or low 2,3-DPG levels which would inhibit normal $O_2$ transport [5,128,164,175].

(2) Hemoglobin

The oxygen-transport protein, hemoglobin (Hb), accounts for 90% of the weight of a mature RBC [175]. It is a spherical tetrameric

molecule comprised of two pair of polypeptide chains, and it weighs about 64,500 daltons [175]. Each of four chains is attached to a heme group, a complex of iron and protoporphyrin. Hemoglobin is normally heterogenous, with HbA predominate in adults and HbF in the fetus. Hemoglobin A consists of two alpha ($\alpha$) chains and two beta ($\beta$) chains. Fetal HbF also contains two $\alpha$ chains but has two gamma chains.

The primary, secondary, tertiary, and quaternary structure of Hb can be affected by chromasomal mutations that change a single amino acid, synthesis, or chemical environment of Hb. Such mutations have often been found to underlie the thalassemias and hemoglobinopathies [2,118,152]. Thalassemia is a hereditary hemolytic anemia that results from decreased or absent synthesis of a globin subunit in the Hb molecule. In contrast, hemoglobinopathies are structural disorders of Hb ususally resulting from substitution of a single amino acid in the globin chain. Thalassemic hemoglobinopathies have been reported where both globin synthesis and structure are abnormal [152]. These Hb mutations are among the most widespread throughout the world and occassionally have a survival advantage in malaria areas or at high altitude [152,175].

f. Oxygen transport

At rest, man consumes about 250 ml $O_2$/min and produces 200 ml $CO_2$/min, amounts that increase nearly 10 times with excercise [175]. Without Hb, plasma transport of $O_2$ would restrict man's activity by 50 times. Rats consume about 10% as much $O_2$/min as humans do at rest.

Evolution has allowed the multicellular organism to provide molecular $O_2$ to mitochondria at a $PO_2$ between 1.0 and 0.1 mm Hg [48].

Oxygen affinity. Each gram of fully saturated Hb binds 1.35 ml $O_2$ [145,175]. The degree of saturation relates to the $O_2$ tension ($pO_2$) which normally ranges from 100 mm Hg in arterial blood to 35 mm Hg in veins. The relationship between $pO_2$ and Hb saturation is described by the oxygen-dissociation curve of Hb (Figure 2). The shape of this curve is determined partly by Hb itself and partly by the RBC environment affected by pH, temperature, ionic strength, 2,3-DPG and other phosphorylated compounds [175].

The oxygen affinity of Hb is described by the $O_2$ tension at which 50% saturation occurs, giving the $P_{50}$ of either Hb or blood. When $O_2$ affinity increases, the curve is shifted left and the $P_{50}$ value is reduced. The curve is normally sigmoidal for Hb which means that Hb can desaturate in a narrow $pO_2$ range corresponding to tissue oxygen needs. The sigmoidal curve results from subunit interaction between the four globin chains. Affinity for each additional bound $O_2$ molecule increases due to conformational changes of Hb, reaching the highest affinity as the fourth and last $O_2$ binding site is filled [175].

g. Hemodynamics

Circulating blood flows within a vascular container composed of large vessels which function as conduits, and a capillary interface for exchange between blood and interstitial fluids [68]. Arteries

contain about 20%, capillaries 10%, and veins 70% of the total blood volume [61].

Viscosity is the property of a fluid to resist flow. Viscosity and Hct have a linear correlation at any given protein concentration [175]. Hyperviscosity tends to decrese venous return while hypervolemia increases venous return; and in most cases of polycythemia, cardiac output is near normal since these two factors offset their effects on venous return [61]. With near normal cardiac output that must handle a blood volume which is sometimes doubled, the circulation time may also double thereby slowing blood flow in polycythemia patients. Arterial blood pressure is usually normal or slightly elevated. In cases of severe polycythemia, the advantage of increased red cell mass and carrying capacity are more than offset by hyperviscosity and deteriorating flow properties of blood [4,65]. A critical viscosity impairs $O_2$ delivery [177]. In such cases, phlebotomy is beneficial by improving hemodynamics in tissues [175].

3. Induced Polycythemia in Wistar Rats

A study at Oregon State University (OSU) in the mid 1970s was designed to determine the effects of methylmercury (MM) on the incidence, latency and distribution of tumors induced in rats by transplacental exposures to ethylurea (EU) and sodium nitrite ($NO_2$) [120]. Partway through this study, rats unexpectantly began to show signs of polycythemia at 2 to 3 months of age [81]. Because the polycythemia was a fortuitous observation, blood values were not obtained for all groups early in the study. The incomplete data on

is minor or nonexistent in rats and humans with neurological disease from MM poisoning [21,30].

The acute oral $LD_{50}$ of mercury in rats is 37 mg/kg for $HgCl_2$, 210 mg/kg for HgCl, 10 to 25 mg/kg for MM, 30 mg/kg for ethylmercury and 60 mg/kg for phenylmercury [165]. Young rats have a higher oral $LD_{50}$ (40 mg/kg) than do aged rats (24 mg/kg) [97]. A 200 mg dose of MM or 1 g dose of $HgCl_2$ taken orally causes death in many adult humans [75]. Tissue levels of MM averaged 2.6 ppm in blood and 27 ppm in brain tissue from humans who died from subacute MM poisoning [10]. Human susceptibility to MM toxicity varies by a factor of 10. Some individuals succomb to a 20 mg body burden, while others tolerate a body burden of 200 mg MM [16]. The minimal, chronic, toxic dose (or threshold dose) in man is about 500 µg MM/day, which causes paresthesia as steady-state blood levels reach 500 µg/l [75,92]. The human fetus experiences toxic effects when maternal blood levels exceed 250 µl/day [75]. Average tissue levels of MM in Minamata disease patients were 0.2 ppm in blood and 60 ppm in liver, and resulted in 20 to 30 mg total body burden [10]. Rats developed neurological disease similar to human Minamata cases after consuming 1 to 2 mg MM/day until a total amount of 15 to 40 mg MM/rat was fed [157]. A safe intake of MM has been calculated to be less than 0.1 mg MM/day which would result in tissue levels of 0.1 ppm in blood, 30 ppm in hair, and a total body burden of 7 to 10 mg of MM [75].

Fetal Minamata disease results from perinatal exposure to MM [141]. The clinical picture closely resembles infantile cerebral palsy

5. Toxicity

Methylmercury compounds were first observed to be potent neurotoxins in man in 1863. Additional cases of MM toxicosis from occupational exposure were described in 1940 and labelled the Hunter-Russell syndrome [104]. Patients had paresthesia, sensory deficits, constricted visual fields, ataxia and dysarthria. Poisoning by MM was considered a hazard only to industrial and research workers until the epidemic of MM poisoning occurred at Minamata, Japan [63]. This epidemic lasted from about 1953 until the early 1960s, and it involved over 1500 poisonings, 46 deaths, and 40 cases of "fetal Minamata disease" through in utero exposure [104,141]. Methylmercury entered the food chain of the Japanese who consumed large amounts of fish in their diets. The local Chisso Corporation dumped as much as 500 tons of MM and inorganic mercury into the Minamata Bay. Aquatic microbes converted much of the mercury into MM which was bioaccumulated in local seafood.

Mercury affects numerous bodily organs and systems. Some effects have been shown in man, while other phenomenal effects occur only in animal models [62]. Mercury compounds have two major target organs regardless of the metallic form, the central nervous system (CNS) and the kidney [25,62,92,102]. The rat again differs from other species since its peripheral nervous system, rather than its CNS, is more sensitive to mercury [111]. This sensitivity is first expressed clinically as rear leg crossover, ataxia and posterior paralysis in rats exposed to sufficient doses of MMC [111]. Clinical renal disease

as high as the mother's, and the fetal erythrocyte has 30% more MM than do maternal RBCs [51,62]. The fetus may actually serve as a compartmental sink which reduces maternal mercury levels compared to nonpregnant females [87].

Metallothionein in the kidney and placenta may protect the renal and fetal target tissues by sequestering mercury [62,166]. Selenium and vitamin E also protect against mercury toxicity [155]. Selenium alters mercury distribution and tissue levels, reduces placental transfer of mercury, and diminishes toxic effects of mercury indirectly and directly [82,133,154,161]. Methylmercury suppresses glutathione peroxidase (GSH-Px) activity which in part is responsible for protecting membrane integrity and cellular macromolecules from oxidation. Selenium nullifies the GSH-Px suppression by MM [30]. Selenium may block mercury from binding to critical sulfhydryl groups or it may bind mercury directly as mercury selenide or bismethylmercury selenide [16].

Absorbed inorganic mercury is eliminated mainly in the urine while MM is primarily excreted in the feces [62,92]. The mechanisms involved are not well known, but exfoliation of renal and intestinal epithelial cells accounts for most o. the mercury elimination in man [62]. The liver conjugates MM with glutathione, and nearly all of this conjugate is reabsorbed and undergoes enterohepatic circulation [16,62]. The hair is another minor site of MM excretion, containing levels about 250 times higher levels than blood [30].

has a longer halflife of about 70 days in man and 20 days in rats [30,102]. The halflife of MM in some human subpopulations ranges from 100 to 200 days, which compounds the toxicological consequences [16]. Female humans and rats have shorter halflives than males, and lactation accelerates clearance [30,102]. Human and rat milk contain only about 1% of the MM dose consumed [51].

Inorganic mercury distributes about equally between blood and plasma, whereas MM is concentrated within the erythrocyte [62,102]. The human RBC contains from 5 to 9 times more MM than plasma, but in rats the RBC levels are elevated 22 to 160 times higher than plasma levels [34,92,102,111]. A dramatic decrease in rat RBC:plasma ratio of MM occurs with increasing age, from 115:1 in 8 week olds to 5:1 in old rats [97]. Part of this effect may be due to increased hemolysis in the older rats. This differential RBC affinity for MM may account for the lower levels of MM in the rat brain when compared to other species [34,102,172]. The brain/blood concentrations of MM are 0.07 in rats, 8 in squirrel monkeys, and over 10 in humans [10,25,102]. Selenium increases this ratio in rats, but reduces clinical neurotoxicity of MM [161].

Methylmercury accumulates in brain and fetal tissues [25,141]. As the carbon chain lengthens, less alkymercurials cross the blood brain and placental barriers [24]. Phenylmercury does not readily cross lipid biomembranes since it is readily cleaved to form benzene and $Hg^{++}$ [16,102]. Halflives of MM in human blood is 172 days [29], and 240 days in the brain [25]. Fetal tissue attains MM levels at least

toxic profiles that result from mercury exposure [16,62,92,103, 104,160]. The following references will emphasize the kinetics of methylmercury chloride (MMC) in humans and the rat. It should be noted, though, that the animal models which more closely resemble human toxicosis by MMC are the rabbit, cat, mouse, and subhuman primate [23,80].

Absorption. Elemental and alkymercurials are lipid soluble and are absorbed through the lungs with about an 80% efficiency [16,62]. However, elemental mercury is poorly absorbed (<0.01%) from the gastrointestinal tract because it forms globules rather than retaining its monoatomic form [62]. Only 7 to 10% of mercuric mercury is absorbed from the intestinal tract, while mercurous mercury is not absorbed, unless oxidized, because of its water insolubility [62,92]. Conversely, MMC is nearly completely absorbed (95% to 100%) from the intestinal tract [24,62]. Skin absorption of mercuric chloride and alkymercurials has been demonstrated at levels which have caused poisoning and death [16,24,62].

Distribution, biotransformation and elimination. The kidney is noted for having the highest affinity for mercury [62]. This organ contains the highest levels of mercuric and methylmercury, and it retains the compounds longer compared to any other tissue [92]. The halflife of mercuric mercury (Hg ++) in man is about 40 days, and about 10 days for the rat [30,92,102]. As the alkyl chain of organomercurials lengthens, the rate of Hg-carbon bond cleavage increases and the halflife in the body is decreased [24,102,122]. Methylmercury

have been recorded from eating non-coastal ocean-going fish [104]. Selenium to mercury molar ratios are usually 1:1 in ocean-going fish, whereas this ratio fell to 1:10 in MM-contaminated fish from Minamata Bay [144]. Nearly all the mercury in fish is MM [130]. Predatory fish can bioaccumulate MM to a level 3,000 times higher than surrounding water levels [155], and the halflife in fish is about 2 years [130]. Contaminated fish from Minamata Bay and from certain North American waterways had levels of MM up to 40 ppm in the muscle [87,104,155]. Humans absorb over 90% of MM present in consumed fish [30]. In addition, humans and other mammals harbor intestinal microbes which convert small amounts of inorganic mercury into MM and visa versa [29,122,143].

Restrictions on environmental pollution of waterways and the prohibition of marketing fish with MM levels > 0.5 ppm have reduced the potential of acquiring Minamata disease [135]. Accidental poisonings from consumption of organomercurial-treated seed grains nave also dwindled since being largely replaced by alternative fungicides [104].

4. Pharmacokinetics

One of the more fascinating aspects of the toxicology of mercury has been the difference in pattern of mercury deposition in organs and tissues by different mercury compounds [102]. The chemical form in which mercury enters the body influences the distribution of mercury as well as the type of toxic response. Variations in dose, age, sex, maternal status, and dietary factors contribute to the widely diverse

amounts up to 150,000 tons per year. This compares to the total human production of about 10,000 tons per year and another 10,000 tons per year released from burning fossil fuels and from other human activites [16]. Mercury cycles through biological organisms, water and the atmosphere where ultraviolet radiation and microbes convert one form of mercury into another [165].

Despite the extensive ecological mercury cycle, exposure to man is generally minimal [62,165]. Average daily intake of mercury for most Americans is about 0.03 mg or 0.04 µg/kg body weight [10,62]. Elemental and inorganic mercury accounts for under 10 µg/day exposure from inhalation, drinking water and food sources in the absence of occupational exposure [16]. Methylmercury accounts for the remainder of human mercury exposure, with most of the MM derived from consumption of fish [16, 104]. Other meat and poultry products contain lower average levels of about 20 ppb MM [16,144]. These exposure levels result in average daily intakes well below the 100 µg/day maximum limit recommended by the FDA [144]. Minor reversible signs of chronic MM toxicity do not appear until intake averages over 500 µg/day, and the appearance of disease depends highly on selenium intake which offsets MM toxicity [74, 75,161]. The fetus, however, is much more susceptible to MM poisoning [141].

Much recent concern has developed over MM exposure and toxicity as a result of Minamata disease and other epidemics of MM poisoning [57,75,87,104,130]. Although fish accumulate large levels of MM, especially in areas of local contamination, no cases of MM poisoning

disagreeable odor, has a density of 4.06 at 25°C, and is slightly water soluble [168].

3. Production, Use and Environmental Sources

Mercury is mined throughout the world mostly in the form of mercuric sulfide (HgS) which is a scarlet-red ore known as cinnabar or vermillion [24]. High-grade cinnabar ore may only comprise 0.02% of the mercury in the earth's crust [24]. The balance of mercury is distributed in rocks and soil at above 0.5 ppm, and in seawater at about 0.03 ppb [165].

The United States used 60,000 flasks of mercury in 1980 and is projected to use about 70,000 flasks (2100 metric tons) in 1985 [30]. In decreasing order, mercury is consumed for manufacture of electrical apparatuses, caustic chlorine, industrial instruments, dental preparations, paints, and pharmaceuticals [30]. Mercury was an important ingredient in many diuretics, antimicrobials, skin ointments, and laxatives [92]. Its pharmaceutical, fungicidal and pesticidal uses are dwindling due to more agents drugs and restrictions on environmental contaminations [30,92]. Mercury is irreplaceable for fluorescent light bulbs, dental fillings, and certain electrical devices [30]. Other minor uses include its application in detonators, catalysts, preservatives in the paper pulp industry, cosmetics, and as fungicides and algaecides in floor waxes, furniture polishes, and laundry preparations to suppress mildew [16,57].

Mercury undergoes massive movement through a global ecological cycle [165]. Mercury degasses from the earth's crust and ocean in

toxic pollutant' by the US Environmental Protection Agency (EPA) [148].

2. Physical and Chemical Properties

Mercury has an atomic weight of 200.6 and an atomic number of 80. The chemical symbol 'Hg' denotes its Latin name, hydra-argyrum [24]. With regard to the toxicity of mercury, three major chemical forms exist: elemental mercury ($Hg^{\circ}$), inorganic salts of mercury ($Hg^{+}$ and $Hg^{++}$), and organic mercury [62,92].

Organomercurials are defined as those mercury compounds having a covalent carbon-mercury bond [24,62,104]. This group is essentially comprised of three subclasses of compounds: the alkoxyalkylmercurials, the arylmercurials, and the alkylmercurials [62,92]. These organomercurial compounds form salts with inorganic and organic acids, and they behave quite differently according to their class [92]. Like the mercuric salts, the organomercurials have very high affinities for sulfhydryl groups ($\log_{10}K=17$) [62,102,165]. Unlike the mercuric salts, organomercurials are highly lipid soluble, especially the short chain alkymercurials which have a lipid:water partition coefficient of about 100 [165].

Depending on the salt, organomercurials may be highly volatile at room temperature. Methylmercury chloride (MMC) has a saturation concentration of 90,000 mg $Hg/m^3$ at 20°C and therefore poses a serious inhalation hazard [16]. Methylmercury chloride is the most common and toxic organomercurial [92,165]. It has a formula weight of 251.1 g/M, melts from its powdery-white crystalline form at 170°C, possesses a

## B. Methylmercury

### 1. Background

Mercury is a liquid, silvery metal which shares its name with a Roman god and has long been attributed magical properties [62]. Aristotle first named this metal "quicksilver", and it has become a part of human history since first used by the ancient Egyptians and Babylonians [30]. Mercury was used to treat nearly every ailment known to man during the past centuries [62]. Its toxic properties were known as early as 1500 BC [165] and its narrow margin for safety was well recognized in the Middle Ages [62].

Several epidemics of organomercurial poisonings have occurred in humans and animals through environmental contamination with, principally, methylmercury (MM). These MM poisonings in humans are called "Minamata disease" (after the first known episode that occurred at Minamata Bay, Japan) and have produced about 8115 cases in humans [30]. Most human exposures to MM result from eating contaminated fish which bio-accumulate MM in their tissues. Several epidemics have also resulted from human consumption of cereal seed grains treated with alkyl-mercury fungicides [62]. Mercury is a nonessential trace element which is ubiquitous in our environment at low levels and is found in all tissues of animals [10,155,165]. Methylmercury is considered the most toxic of the mercury compounds due in part to its high lipophilicity [165]. Methylmercury is listed as a 'priority

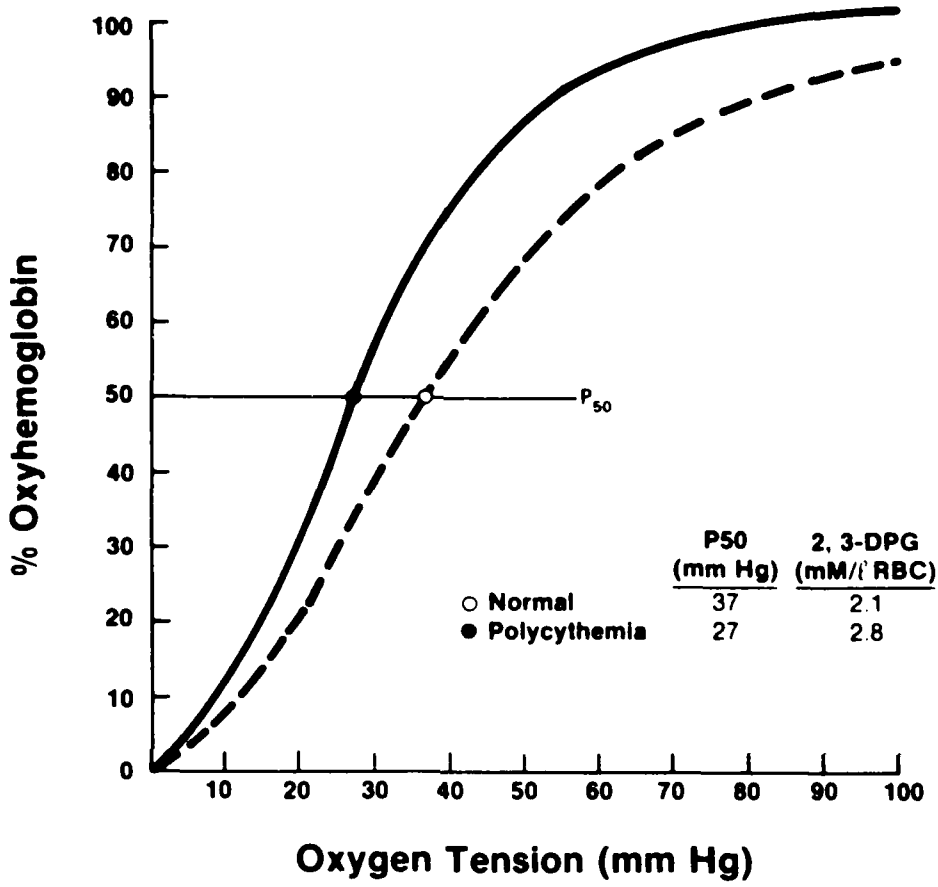


Figure 4. Oxygen-Erythrocyte Dissociation Curve for a Polycythemic Wistar Rat.

The polycythemic rat exhibited a 10 mm Hg left shift in the $P_{50}$ value of its erythrocytes when compared to the control. No significant difference was detected in levels of 2,3-diphosphoglycerate (2,3-DPG). (Data from Koller, 1984, ref no. 85).

While no specific percent Hct was defined for diagnosis of the 36 polycythemic rats, the mean Hct was 75% compared to 56% for littermates and 44% for control rats [81]. Onset began 1 month of age, peaked at 2 to 3 months, and appeared occasionally after 4 months of age. Water deprivation tests provided evidence that polycythemia was not due to hemoconcentration; however, red cell mass measurements were not performed to distinguish relative from absolute polycythemia. Pathological examinations revealed hyperplastic marrow and megakaryocytosis. White and red blood cell counts were elevated.

Analyses showed that whole blood $O_2$ affinity was increased (Figure 4) in polycythemic rats despite normal values of 2,3-DPG and Met/sulf/carboxy Hb, identical hemolysate $O_2$ equilibriums (an indicator of Hb affinity for $O_2$), and identical electrophoretic spectrums of Hb performed at pH 8.6 [85]. The mean $P_{50}$ value of control rats was about 34 mm Hg vs 24 mm Hg in polycythemic rats [85]. Hemolysate $O_2$ affinity was not measured. The possibility that the erythrocytosis was a compensatory response to an increased $O_2$ affinity was acknowledged. The elevated WBC counts, megakarocytosis, and splenomegaly would be atypical, but possible, findings with high $O_2$ affinity erythrocytosis [99]. Platelet counts, red cell mass values, and Ep levels were future analyses deemed necessary to clearly define whether this disease in rats actually models PV in humans [81].

nistered either by gavage on days 17, 18 and 19 of gestation or by EU in feed and $NO_2$ in drinking water from day 14 of gestation until parturition. Two of these 5 groups received either 100 and 50, or 50 and 25 mg/kg EU and $NO_2$ dissolved in distilled water. The other 3 groups received 6.36 and 2, or 3.18 and 1, or 1.59 and 0.5% EU in feed and g/l $NO_2$ in drinking water. Additional nonexposed control groups and groups fed EU and $NO_2$ without MM were also studied. Only 3 groups of offspring developed polycythemia at incidences of 14 to 24% in the original report [81] which actually was 24 to 41% at the study's completion [85]. Polycythemia only occurred in groups of offspring exposed to all three chemicals (MM,EU,$NO_2$), except in each of the groups exposed to MM and high doses of ENU [120,121]. Ten pups were weaned from the high dose groups, and they experienced early death from neurogenic tumors [119,120]. The average number of pups weaned per female fed 10 or 15 ppm MM was reduced to 6.1 and 0.9 [121]. Methylmercury synergistically enhanced the transplacental toxicity of ENU by reducing reproductivity of dams and survival of progeny [119]. The latency of neurogenic tumors was reduced and incidences of schwannomas and ependymomas increased in progeny [120]. Ethylnitrosourea alone was most toxic in the second trimester, while MM plus ENU were most harmful in the third trimester [121]. Studies with $^{14}C$-ENU administered to dams on day 20 of gestation showed no difference in ENU levels or halflives in the fetal brain in MM-treated vs non-MM-treated groups [121].

the polycythemia, coupled with high mortality at an early age in some exposure groups, prevented a complete analysis of the disease. The results indicated, however, that the chemical-induced polycythemia in these rats shared many features with human PV, including elevated Hcts, increased megakaryocyte, WBC and RBC counts, marrow hyperplasia, and splenomegaly [81].

Several unsuccessful attempts were later made to reproduce the polycythemia with animals other than those from the closed colony of Wistar rats used in the initial study. Rabbits, mice, Fisher 344 rats, and Charles River Wistar rats were tested [5,85,121]. The original investigators did repeat the polycythemia in a few rats from the closed colony, but further diagnostic tests were not performed. The induction of polycythemia solely in these Wistar rats may indicate a familial susceptibility to the chemical-induced disease. The colony of Wistar rats at OSU was terminated in the late 1970s, but a remnant population was moved to the University of Idaho for further studies. Of the 11 remaining rats from the original closed colony, only 2 males and 2 females were fertile. The offspring from these four Wistar rats were used in subsequent studies aimed at fully characterizing the polycythemia as reported in this dissertation.

In the original study 6 groups of weanling Wistar rats were exposed for 13 weeks to MM-chloride dissolved in corn oil and mixed in the feed. They were mated after 10 weeks of MM exposure, and 5 groups received ethylnitrosouresa (ENU) precursors during the third trimester of gestation. Ethylurea (EU) and sodium nitrite ($NO_2$) were admi-

with ataxia and mental retardation [16]. Oftentimes the mothers of such children did not exhibit any signs of MM toxicity during pregnancy [141]. The human fetal brain is the target organ during pregnancy, since it is 2 to 5 times as sensitive to MM exposure compared to adult brains [104].

Human teratological effects were only reported in a few Minamata Bay cases, but defects were not significantly greater than normal [87]. A multitude of teratological defects have been induced with MM exposure in pregnant animals [41]. Methylmercury is most toxic to the fetus during the second trimester of gestation [87,121]. Behavioral abnormalities have also been reported in animals exposed to MM in utero [43,141]. Other effects caused by perinatal MM exposure include alterations in the blood-brain barrier, neurotransmitter dynamics, carbohydrate metabolism, and ornithine decarboxylase activity [9].

The earliest signs of MM toxicity in adult rats are anorexia and weight loss [102,165]. Chronic exposure to levels of MM higher than 0.25 mg Hg/Kg/day produces neurotoxic signs, decreased Hct and Hb values, renal lesions, and increased mortality in male rats which are more prone to these effects than are females [111]. Adult male rats retain more MM than do females [160]. Tissue levels of MM were also generally greater in men than women patients with Minamata disease [76].

Reproductive toxicity is readily produced in rats exposed to MM, but no adverse effects on reproduction have been observed in humans with Minamata disease [21,165]. Methylmercury has been shown to be

mutagenic in plants and in lower animals, and possibly in cultured human lymphocytes [16,24]. Studies have shown that MM may act as a co-carcinogen or as a promoter, or possibly as a carcinogen in mice [19,120]. Elemental mercury was carcinogenic following IP or IM injections in rats [36].

Perhaps the most sensitive indicator of MM toxicity is the immune system [84]. Levels as low as 1 ppm in the diet caused significant reductions in primary immune responses of mice and increased mortality of mice exposed to encephalomyocarditis virus [82]. The developing immune system, including the entire hematopoietic system, may be especially susceptible to MM intoxication [82,154,165].

Mode of action. The primary mode of action of inorganic and organic mercury is thought to be interference with membrane permeability and enzyme reactions by binding of mercury to sulfhydryl groups [92,123]. Biological sites of highest MM affinity include the fetal blood and brain [141], peripheral sensory neurons and cerebellar granular cells [123], renal proximal tubular epithelium [26,111], and hemoglobin [34,172]. Rat Hb has twice the affinity for MM compared to human Hb [34]. Methylmercury is a strong protein denaturing agent which binds cell membranes and penetrates intracellularly to directly degrade organelles and cytoplasmic or nuclear proteins [25].

6. Pathology

Renal pathology. Mercuric mercury ($HgCl_2$) is more toxic to the kidney than are the organomercurials (MM), but the pathology induced by each is very similar [26]. The pars recta portion of the proximal

tubules appears to be most sensitive to MM toxicity, while other tubular sections and renal glomeruli are usually spared [26]. The pathological changes are nonspecific and are general cytological changes related to cellular injury and necrosis [26]. In utero exposure to MM caused similar renal lesions in rat proximal tubular cells with occasional glomerular lesions. Besides degeneration of proximal tubules, hyperplasia of distal tubules suggestive of pre-malignant changes have been reported in rats exposed to MM in utero [26]. Small, multifocal cortical cysts are often observed in kidneys from rats exposed to MM in utero, and they develop from dialated proximal tubules [111].

Neuropathology. Histopathology reveals demyelination and vacuolization of dorsal nerve roots and peripheral nerves [111], cerebrovascular lesions [146], gliosis, and atrophy of the cerebellar granular cells [123]. Some pathological lesions in MM toxicosis partly result from metabolic disruptions caused by the damaged BBB [25]. In utero exposure to MM results in more widespread distribution of the chemical and lesions in the brain [25,141]. Brain atrophy, hypoplasia of cytoarchitecture, and malformed neurons were additional lesions observed in fetal Minamata disease but not observed in infantile and adult cases [25,141]. Animal studies have also shown delayed granular cell migration, abnormal increases in numbers of neuronal lysosomes, and disruption of neuronal endoplasmic reticulum and mitochondria [141]. Exencephaly and hydrocephaly were also reported in rats exposed to MM in utero [141].

## C. Ethylnitrosourea

### 1. Background

Ethylnitrosourea (ENU) is one compound from a group of chemicals designated as N-nitroso compounds. Many of these agents are classified as genotoxic chemical carcinogens [170]. Toxicity of N-nitroso compounds was first reported in the late 1930s, but it was not until 1956 that Magee and Barnes demonstrated the potent hepatotoxic and carcinogenic effects of these compounds [101,124]. Druckery et al later described the organotropic effects of 65 N-nitroso carcinogens administered to rats [38]. Druckery et al also reported the first cases of cancer resulting from in utero exposure to N-nitroso compounds [37]. Since then over 30 chemicals have been shown to be prenatal carcinogens [31].

Ethylnitrosourea displays a tropism for developing nervous tissue of rats exposed perinatally [78]. Until the advent of ENU-induced neurogenic tumors in rats, no suitable animal model existed to allow the study of neural tumors in children [78]. Today it is generally recognized by many scientists that N-nitroso compounds pose a significantly potential hazard to human health [35]. Although no conclusive evidence in humans is available yet, the International Agency for Research on Cancer (IARC) recommends that N-nitroso compounds be regarded as carcinogenic in humans [71,124].

2. Physical and Chemical Properties

Ethylnitrosourea is an alkyl-acyl-nitrosamide with a molecular formula of $C_3H_7N_3O_2$ [78]. Its molecular weight is 117.13 g/M, it is slightly soluble (1.3%) in water, and it is yellow-pink in its pure crystalline form [168]. ENU does not occur naturally. It was first synthesized in 1919 in Germany from the reaction of ethylurea (EU) and nitrous acid ($HNO_2$). Sodium nitrite ($NO_2$) and EU combine at the optimum pH of 3.4 to form ENU both in vivo and in vitro [107, 112,176]. As pH increases, yield of ENU decreases [107]. The stability of ENU in the body or in other aqueous solutions improves with acidity. At physiologic or alkaline pH, ENU spontaneous decomposes by hydrolysis to form electrophilic isocyanate (O=C=N-H) and ethyldiazohydroxide (Et-N=N-OH) compounds [126,139]. Any nitrosable secondary or tertiary amine may combine with a nitrosating agent to form N-nitroso compounds [35]. In contrast to the nitrosamide compounds like ENU, nitrosamines do not spontaneously decompose into electrophiles until they are enzymatically oxidized [170].

3. Production, Use, Environmental Sources

N-nitroso compounds are produced commercially on a limited basis for research, and recently these compounds are being designed as cancer chemotherapeutic agents [19,71]. N-nitroso compounds are ubiquitous in our environment [95,12,120]. Air, water, food, automobile interiors, cosmetics, and smoke contain these chemicals [71]. Tobacco smoke and beer account for the highest levels (1ppb) [176]. Occupational exposures are highest in tannery, rubber, and propellant

industries [71]. In addition, nitrosable and nitrosating compounds are widespread at sometimes high levels in our environment [35]. Drugs, detergents, pesticides, herbicides, food preservatives, vegetables, preserves, pickles, fish, bread, cheese, cereals, animal feeds, and surface water contain nitrosable amines or amides [71,112]. Nitrites are used to preserve meats at concentrations up to 125 ppm [96]. Nitrate is used as fertilizer, and it can be reduced by bacteria in the soil or intestines to form $NO_2$ [35]. Many leafy vegetables concentrate nitrate under favorable soil and water conditions [176]. Human saliva contains nitrosamines [158] and nitrate which is reduced to nitrite (10 mg/day) by oral microflora [170,176]. Some nitrosated compounds can trans-nitrosate amines and amides [71]. Normally, small amounts of N-nitroso compounds can be detected in human blood, especially after meals [178].

4. Pharmacokinetics

N-nitroso compounds are absorbed, enter the blood stream, and cross the placental and blood-brain barriers [71, 170]. The nitrosamines require enzymatic oxidation, but nitrosamides spontaneously decompose at physiological pH into electophilic products. The halflife of ENU is <8 min in the body and 5 to 6 min in the blood [139]. The ethyldiazonium ion derived form ENU ethylates all bodily tissues to a similar degree which denotes its uniform distribution [139]. In contrast, the nitrosamines often alkylate tissue near the site of enzymatic activation such as in the liver.

The ethyldiazonium ion and isocyanate react with cellular nucleophiles such as DNA. Isocyanate preferentially reacts with lysine and arginine groups on proteins, especially Hb [66]. Ethyldiazohydroxide preferentially binds oxygen sites in cells [27]. Conjugation by glucoronides and amino acids, and hydroxylation tends to detoxify and eliminate these by-products [126].

5. Toxicity

Nitroso compounds are noted for their potent carcinogenic effects [71]. Ethylnitrosourea is carcinogenic in virtually all living systems, even in vitro [139,170]. Short chain alkylureas are leukemogenic [124]. The type of leukemia produced by ENU varies widely with strain or species, dosage regimen, age, sex and other factors [124,125,139]. Besides being carcinogenic, ENU is mutagenic, teratogenic, and toxic in other regards depending on exposure conditions [35]. The acute oral $LD_{50}$ in rats is 300 mg/kg [71].

Adverse effects of ENU and other N-nitroso compounds follow a linear dose response curve which gives little evidence of a no-effect threshold dose [124,134]. Single small doses are additive and summation of dose-related effects occurs [134].

Ethylnitrosourea is recognized as the most potent point mutagen and perinatal carcinogen known [105,139]. A single dose of ENU administered to a pregnant rat can elicit neural tumors in 100% of the offspring [78,139]. The cellular target of ENU is the neuro-ectodermal plate cells in developing rat brains [75,78]. Schwann's cells and glial cells are transformed to produce peripheral or central

nervous system tumors in rats exposed to ENU between gestational day 11 and 30 days postnatally [78,79,139]. Before day 11 the rat's chorioallantoic placenta has not developed, which may help explain the absence of carcinogenic effects when ENU is administered before day 11 [11,79]. Ethylnitrosourea is most acutely toxic during the second trimester, but most carcinogenic during late gestation and shortly after birth [35,121]. The rat fetus is 50 times more sensitive than the adult to neural tumor induction [105,124]. Neurogenic tumors occured in the subsequent $F_1$ and $F_3$ generations of rats exposed to ENU during pregnancy [162].

Metals such as lead and methylmercury, and antioxidants have altered the genesis of neural tumors in rats [120,170]. The precursors of ENU (EU and $NO_2$) have not by themselves produced tumors in rats exposed perinatally [96,120]. However, human epidemiological studies have shown correlative, but nonconclusive, links between high $NO_2$ levels and the occurance of certain cancers [170].

The Donyru strain of rat is considered most sensitive to the leukemogenic effects of ENU [125]. Retroviral particles have been continuously produced in vitro following ENU treatment of mouse cell lines [137]. An etiological role for the retrovirus in rat erythroleukemia has not been shown [66,125].

Transplacentally induced neoplasia may be responsible for many childhood tumors [39,78,105,124]. Human leukemia and neurogenic tumors comprise 50% of cancer deaths in children, and no doubt these neoplasias arise in utero [105]. Because many N-nitroso compounds

have proven carcinogenic in every species tested, it is difficult to believe that humans are exceptional.

Mode of action. Genotoxic chemcial carcinogens are thought to alter the structure of DNA in chromatin of target cells to begin the multistep processes of transformation and tumorigenesis [64,139,170]. Studies have shown that ENU ethylates the oxygens of phosphodiester bonds in DNA nucleotides to the greatest extent; however, this binding causes little cellular damage [139]. Ethylation (Et) of the DNA base, guanine (G), correlates best with development of cancer [27,139]. While both the N7 and O6 sites of guanine are ethylated to the greatest extents, the O6-EtG persists in developing brain tissue but not in the liver or kidney [27,139]. A mutation can occur if the DNA replicates prior to repairing the O6-EtG [139]. Normally guanine base-pairs with cytosine, but O6-EtG is misread as adenine and base-pairs with thymidine. This causes a substitution of 'T' for 'C' in the daughter replicate DNA strand.

Immunity may play a role in nitroso-compound induced neoplasia [153]. Lymphotoxin prevented DEN-induced tumors in vivo [136]. While ENU-induced tumors are sensitive to natural killer (NK) cells in vitro, these tumors appear to be "immunologically privileged" within the nervous system and thus evade NK cells [33,79]. Lifetime studies on rats that had suppressed or enhanced immunity showed no effects on ENU-induced tumorigenesis [110,134]. Butylnitrosourea has been shown to be immunosupressive and toxic to marrow cells [66]. Many studies that reported immune suppression by N-nitroso compounds were conducted

at high levels of exposure [180]. Carcinogenesis may not necessarily result from suppression of the immune surveillance mechanism [10, 79,180].

6. Pathology

Perinatal ENU exposure in rats causes nearly a 100% tumor incidence with 90% neurogenic tumors [73,78]. About 75% of the tumors were located in the brain, and the remainder in the peripheral nervous system (PNS). Over 90% of the CNS tumors were of glial origin [78]. The CNS tumors were located mostly in the periventricular areas of the lateral ventricles. The PNS tumors were formed primarily in the trigeminal nerves and lumbosacral plexus [78]. Neuroblastomas arose earliest, followed by gliomas and later by subependymal-plate (neuroectoderm) tumors [73]. A prevalence of either PNS or CNS neoplasms may be produced by altering the strain, age, sex and dose schedule of N-nitroso exposure [78].

In previous experiments with Wistar rats that developed polycythemia, similar types of ENU-induced neurogenic tumors in progeny were produced as reported above [73,78,120]. About 90% of the offspring exposed to the highest dose of ENU developed neurogenic tumors and had about half the survival time of control offspring. At the dosages used in this study, neurogenic tumors developed in about 60% of the offspring with a latency of about 400 days [120]. Most tumors were located in the CNS and were diagnosed as primitive neuroectodermal tumors, oligodendrogliomas, meningiomas, and schwannomas. Ependymomas arose only in offspring also exposed to MM, and

schwannomas occurred more frequently in these groups. Aged Wistar rats spontaneously develop up to 6% similar types of neurogenic tumors as induced by ENU [156].

D. Animal Model Considerations

Alexander Pope stated, "The proper study of mankind is man". Since this approach is often impractical or impossible, bioassays are relied upon to provide data that can be extrapolated to man.

The Surrogate Species Workship was sponsored by the EPA in 1981 to resolve issues regarding the selection of animal models for ecotoxicological testing [83]. Ten criteria were deemed necessary to develop a good surrogate for study of human disease: 1) the model should accurately produce the disease or lesion; 2) it should be widely available and 3) exportable; 4) genetic disease models should be developed in a polytocous species; 5) the animal should be large enough for multiple samples, and 6) able to be housed in standard animal facilities, and 7) easily handled; 8) an ideal model would be available in multiple species; 9) the model should survive long enough to be usable; and 10) the model should be relatively free from spontaneous diseases other than those used for modeling.

The rat has been used for research since the early 1900s when the Wistar Institute began extensive studies on albino rats, Ratus norvegicus albinus [127]. In toxicological studies the rat is advantageous because it is omnivorous and will ingest powdered diets that include high levels of test material. Recently the rat has shown usefulness as a model for immunotoxicity assessment [86]. Many

physiological and anatomical similarities between rats and humans justify the use of rats in toxicological evaluations. However, qualitative and quantitative differences affect interspecies extrapolation [127].

The major hematological differences between humans and rodents include higher RBC counts, lower RBC volumes, and relatively more lymphocytes in rat blood [127]. Bilirubin, cholesterol, creatinine, albumin, and uric acid levels are lower in rats compared to man, while serum enzymes are generally double or higher in rats [142].

A hematotoxicological difference exists between the RBCs and hemoglobin (Hb) of humans and rats in relation to their affinity for methylmercury [114,172]. In rat RBCs the MM is mostly bound to Hb, whereas MM is primarily bound (46%) to glutathione in human RBCs [34]. Glutathione levels are similar in human and rat RBCs. Rat RBCs release 8 times less MM than do human RBCs. The higher affinity of rat RBCs for MM is difficult to explain, but may be related to the fact that rat RBCs have twice as many external cysteine residues on their Hb molecules [34]. Although the rat may not be a good model for studying toxicokinetics of methylmercury in man, it can serve as a good human model for placental transfer, fetal mercury toxicity, perinatal ENU toxicity, and for hemodynamics and regulation of hematopoiesis [34,72,73,78,111,127,140,151].

# III. MATERIALS AND METHODS

## A. Chemicals

The major chemicals used in this study were methylmercury chloride (Alpha,No. 8036), ethylmercury chloride (Alpha, No. 37114), phenylmercury chloride (Alpha, No. 37113), mercuric (II) chloride (Alpha, No. 89046), ethylurea (Aldrich, 97% pure, Cat. No. ES 100-7), $^{3}$H-thymidine (New England Nuclear, Boston, MA, 6.7 Ci/mM, NET 512), Hydrocount sodium chromate (New England Nuclear, NEZ-030), sodium thioglycollate (Difco Laboratories, Cat. No. 0233-15), Hank's Balanced Salt Solution (Gibco Diagnostics, Cat. No. 450-1201), L-cysteine (Sigma, Cat. No. C-755), sodium nitrite (Aldrich, 97% pure, Cat. No. 20,783-7), RPMI Medium (Gibco), butylurea (Aldrich, No. BIO, 300-4), N-nitrosodiethylamine (Sigma, No. 0258), N-nitroso-N-ethylurea (Sigma, No. N-3385), and STAT stain (VWR Scientific, No. 48662-177).

## B. Biological Reagents

Biological reagents used in this study were bovine serum albumin (Sigma, No. A4378), alkaline phosphatase (Sigma, No. P5521, Type VIII-S), p-nitrophenyl-phosphate substrate (Sigma, No. PI04-40), Freund's complete adjuvant (Cappel Laboratories, No. 0000-0890), goat anti-rat IgG antibody (Pel-Freeze Biologicals, No. 0774), rabbit anti-SRBC antibody (Microbiological Associates Bioproducts, No. 55-402J), keyhole limpet hemocyanin (Calbiochem-Behring Corp., No. 230145), sodium caseinate (ICN Nutritional Biochemicals, No. 102896), powdered milk (Carnation Dry Milk, 12 quart carton), alkaline phos-

phatase-conjugated rabbit anti-rat IgG antibody (Zymed Laboratories, No. 20729), concanavalin A (Sigma, No. C2631), HT-2 cells (ATCC Cell Lines, No. TIB160), and YAC-1 cells (Washington State University).

C. Experimental Animals

Wistar rats originating from a closed colony at Oregon State University were transferred to the University of Idaho in 1980 and used in all experiments. In a separate experiment to test alternate strain or species susceptibility to chemical-induced polycythemia, inbred Lewis rats, outbred Sprague-Dawley (S-D) rats and Swiss-Webster mice were obtained from Washington State Unviersity, Department of Laboratory Animal Resources. The animals were housed 4 per cage in racks of stainless steel hanging wire cages, except for mice and pregnant female rats and their pre-weaning age pups, which were housed in polycarbonate cages containing heat-treated hardwood shavings. Feed and water were available ad libitum throughout the experiments. Purina rodent chow (Catalog No. 5012) was purchased commercially and used as the basic diet in all experiments. The rodent chow was obtained in the unpelleted meal form and appropriate concentrations of chemicals (e.g., mercury compounds) were added prior to mixing. Pelleted rodent chow was fed to groups of rats not being exposed to mercury. New batches of feed were mixed for each experiment. The drinking water was deionized and was provided in clear glass water bottles (16 oz) with rubber stoppers and stainless steel controlled-flow siper tubes. The animal rooms were maintained on a 12-hour on/off automatic light cycle and temperature was controlled

# IV. RESULTS

## A. Onset and Incidence of Reproduced Polycythemia

The first case of polycythemia occurred at 6 weeks of age. Out of the 27 total rats with a Hct greater than 60%, 18 developed polycythemia by 12 weeks of age with a mean onset of 8 weeks (Figure 5). The 9 remaining rats (6 males and 3 females) developed polycythemia between 3 and 8 months of age. Of 20 polycythemic rats which were not sacrificed for early special tests, 4 males and 2 females had their Hcts regress to normal 3 to 6 months after onset. Polycythemia regressed in 2 of 11 rats with early onset and 4 of 9 with delayed onset. The other 14 rats had Hcts that persisted and became higher until death from 2 weeks to 6 months after onset.

Five of the 8 treatment groups produced polycythemic rats (Table V). Rats in group 2 were treated to duplicate the original study, and they had the highest incidence (42%) of polycythemia. Male rats were affected at twice the rate of females. Groups of rats exposed to MM alone or in combination with one ENU precursor also developed polycythemia. Of these groups (4,5,6), the rats from group 5 (exposed to MM and $NO_2$) had the highest incidence at 23%. Rats in group 8 received double the exposure to ENU compared to group 2 but developed a lower incidence (32%) of polycythemia.

Of the 140 progeny from the 5 groups developing polycythemia, 27% (16 of 59) of the rats were male while 14% (11 of 81) were female. Although an elevated Hct greater than or equal to 60% was used to

5. Susceptibility of Alternate Strains and Species

The induction of polycythemia in non-Wistar strains or species was tested to determine if this closed colony of Wistar rats was uniquely susceptible to the transplacental chemical exposures. Four weanling outbred Sprague-Dawley and 4 weanling inbred Lewis rats were treated for 13 weeks with 10 ppm MMCys in the diet. At 10 weeks they were mated with like-strain males and gavaged with 1.0 ml of 0.17 mM EU and $NO_2$ on days 17, 18 and 19 of gestation. Six Swiss Webster female mice were also treated with 10 ppm MMCys for 13 weeks, but they received 1g/l $NO_2$ in drinking water and 0.32% EU in feed during the third trimester of gestation (week 13 of MM treatment). Offspring were observed daily and Hcts were measured weekly to detect any polycythemic rodents.

F. Statistical Analysis

The data were evaluated with an analysis of variance (ANOVA) program from a Statistical Analysis System (SAS, Helwig and Council, 1979) package on the Univesity of Idaho's IBM P3887 computer system. Least squares means analyses were performed to detect significant differences between groups which first showed a significant Fisher's Test value in the ANOVA. Statistical interactions between treatments, ages, sex, days of experiment, or other variables were analyzed to better determine effects due to treatments.

surgically removed and grafted to subcutaneous tissue on the back of another rat. Grafts were observed daily for 4 weeks for signs of rejection.The degree of acceptance or rejection of grafts could indicate how closely related individual rats are within the closed Wistar colony. Inbreeding tends to express recessive inheritable traits [53].

4. Induction by Chemical Analogs

The effect of nitroso analogs on the development of polycythemia was tested by gavaging pregnant dams with equimolar (0.17 mM/day) levels of ethylnitrosourea (ENU) precursors , diethylnitrosamine (DEN), and butylnitrosourea (BNU) precursors, on days 17, 18, and 19 of gestation. Dams were previously exposed to 10 ppm MMCys in the diet for about 13 weeks. Dams from each of the three groups received 1 ml per day of the specific N-nitroso coumpound. Offspring were observed daily and hemotocrits were measured weekly to diagnose polycythemia.

In another experiment, 12 weanling female Wistar rats were equally divided into 4 groups, and each group was fed either 10 ppm of MMCys, ethylmercury cysteine (EMCys), phenylmercury cysteine (P-MCys), or mercuric chloride cysteine (HgCl-Cys). The rats were equally divided into 4 groups, and each group was fed either 10 ppm of MMCys, EMCys, PMCys, or HgCl-Cys. The rats were mated at 10 weeks and gavaged with 0.17 mM of ENU on days 17, 18 and 19 of gestation. Offspring were observed and bled for Hct measurements to determine the incidence of polycythemia induced by the various mercury analogs.

with or without 1.0 µg/ml of concanavalin A (Con A), and the cell-free supernatant was harvested and analyzed for IL2 content. The IL2-containing supernatant was serially diluted and added in 100 µl aliquots to 96 well culture plates containing 100 µl ($5x10^3$ cells) of the IL2-dependent T lymphocyte clone, HT-2 cells. The HT-2 cells and IL2-containing supernatants were incubated for 18 hours, pulsed with $^3$H-thymidine for 4 hours, harvested onto glass fiber filters and counted on a scintillation counter. Control cultures included naive HT-2 cells, non-Con A-stimulated spleen cell supernatants, media only, Con A-stimulated HT-2 cells, and an IL2 standard. The results were recorded as mean counts per minute (cpm).

Production of $PGE_2$ by resident peritoneal adherent cells from each rat was measured by a radioimmunoasay (RIA) technique. Briefly, resident peritoneal cells were collected by lavage, washed, counted on a Coulter counter, and diluted to $1.5x10^6$ viable cells. Non-adherent cells were removed by rinsing following a 90-minute incubation. $PGE_2$ synthesis was stimulated by addition of LPS for 20 hours after which supernatants were collected and stored at -20°C until analyzed by a double antibody RIA technique utilizing rabbit anti-PGE-BSA serum and goat anti-rabbit IgG. The lower sensitivity limit for detection of PGE2 was 10 pg/tube. The results are expressed as pg $PGE_2$/ml of supernatant.

A host versus graft (HVG) analysis was also conducted on 4 male Wistar rats from different litters to test the degree of histo-compatibility. The dorsal layer of skin from the ear of each rat was

microcuvette trays were coated with KLH and serum samples were added at various dilutions. After incubation and washing, goat anti-rat IgG, conjugated to alkaline phosphatase, was added to each well. Following a second incubation, a substrate for alkaline phosphatase was added to each well and the color reation was quantitated spectrophotometrically on the Gilford Auto-analyzer. The values were recorded as absorbance at 405 nm. Background absorbance was considered to be 0.30 based on average absorbance of dilulent without ser .

DTH reactivity was used to assess in vivo cell-mediated immunity in rats. Rats were sensitized sc with bovine serum albumin (BSA) emulsified in Freund's complete adjuvant (FCA), and challenged seven days later by injecting the footpad with heat-aggregated BSA. A solution of BSA in saline was mixed 1:1 by volume with FCA, and 100 ug BSA in a 100 µl aliquot was injected sc at the base of the tail. Seven days later, the left rear footpad was challenged with 100 µl of 2% heat-aggregated BSA. The right rear footpad was sham-injected with sterile physiological saline. Twenty-four hours later, footpads were measured for swelling using an electronic digital micrometer. The thickness of the saline-injected footpad was subtracted from the BSA-injected footpad to determine the DTH reaction. The data were recorded as mm swelling.

The assay for IL2 production required spleens that were removed aseptically from each animal and forced through stainless steel screens to obtain single cell suspensions which were counted and diluted to $1x10^6$ cells/ml. One ml aliquots were incubated 2 hours

Immunocompetence. An immunological analysis was conducted on 8 control rats and 12 offspring of dams treated with MM, EU, and $NO_2$. Sex and age effects on immunity were analyzed. Humoral immunity, cell-mediated immunity, NK cell activity, PGE, and interleukin 2 (IL2) were analyzed as reported by Exon and Koller [47,86]. The one-rat model for immunotoxicology testing was used and individual procedures are described below and in Table IV.

Splenic NK cell activity was determined in a 4 hr $^{51}Cr$ release assay. Single cell suspensions were prepared from rat spleens in complete medium and the RBCs were lysed by hypotonic shock. Adherent and phagocytic cells were removed by incubating the cells on nylon wool columns with a subsequent one-hour incubation in tissue culture flasks. YAC-1 tumor cells were labelled with $^{51}Cr$ and used as NK targets. The target cells were added to 96-well flat-bottomed microtest plates containing varying concentrations of NK effector cells. After an incubation period of 4 hours the cell-free supernatant was collected from each well and counted on a gamma counter. Specific $^{51}Cr$ release from lysed target cells was calculated by the formula:

$$\frac{\text{experimental release} - \text{spontaneous release}}{\text{maximum release by 2\% SDS} - \text{spontaneous release}} \times 100\% \text{ cytotoxicity}$$

The antibody response to KLH was analyzed by a modified ELISA (enzyme linked immunosorbent assay) procedure on a Gilford PR50 Autoanalyzer. Briefly, serum samples were collected from KLH-injected rats by cardiac puncture and stored at -20°C until analyzed. ELISA

3. Pathogenic Investigtions

Diagnostic scheme. Figure 1 outlines the algorithm used to diagnose polycythemic rats in this study [4,5]. The diagnosis should relate directly to the pathogenesis. Numbers of megakaryocytes and areas of marrow cells per cross-section of femoral marrow cavity were measured by stereology (Bioquant Digitizing Morphology computer program) to assist diagnosis of possible primary polycythemia. Four replicates of marrow cross-sections were measured per rat. Further Hb analyses (hemolysate $O_2$ affinity) were intended if the rats had polycythemia without apparent secondary causes as originally reported [94], and if sufficient numbers of polycythemic rats were available.

Mercury levels in tissues. Organs and tissues were collected from dams, pups, and polycythemic and control offspring for analyses of mercury content. Tissue was frozen until analyzed by atomic absorption spectrophotometry.

Transmission of disease. Horizontal and vertical transmission of polycythemia was studied. In one experiment, 2 week old Wistar rats were injected with 0.25 ml each of blood from a polycythemic rat. Two males and 2 females received a single sc injection, and 2 males and 1 female received single ip injections. The same number and sex of littermates served as controls and received equal amounts of saline. Vertical transmission was tested by breeding 2 male and 2 female polycythemic rats and observing for polycythemia in the 2 litters of offspring.

and control rats for clinical pathology studies. Urine pH, protein, glucose, ketone, bilirubin, blood and urobilinogen were determined with Multistix reagent strips (Ames Div, Miles Laboratories). A small animal profile was run on the serum samples at Washington State University's Veterinary Clinical Diagnostic Laboratory. Tests included blood urea nitrogen (BUN), creatinine, calcium, phosphorus, total protein, albumin, alkaline phosphatase, alanine aminotransferase (GPT), glucose, and cholesterol. The same laboratory was used to perform blood gas analyses on a few polycythemic and control rats. Arterial blood pH, $PCO_2$, $PO_2$, bicarbonate, total $CO_2$, $O_2$ saturation, and base excess were determined.

d. Dose and time-response studies

In order to test the effect that duration of MM exposure had on the incidence of polycythemia in offspring, dams were treated in a separate experiment with MM for 3, 4, 5, 6, 7 or 10 weeks prior to breeding and during gestation. Dams were gavaged with 0.17 mM each of EU and $NO_2$ on days 17, 18 and 19 of gestation. The hematocrits of offspring were measured weekly.

Another experiment was performed to test the dose-response relationship of MM treatment on dams and the incidence of polycythemia in offspring. A log dose design was implemented where dams were fed MM for 13 week at 2, 4, 8, 10, 12, 16, 20 and 24 ppm in the diet (log dose = .3, .6, .9, 1.0, 1.1, 1.2, 1.3, 1.4). Hematocrits of offspring were measured weekly.

microscopically for white cell differential counts, morphology of RBCs, and presence of reticulocytes or leukemic cells.

b. Genetic monitoring

Four male and female Wistar rats from the original colony were fertile and eventually produced the 53 weanling females needed to repeat the polycythemia. Male Wistars from the same parental lines were chosen for mating with the 53 females so that the 4 genotypes would appear in all offspring but at different percentages. Such a breeding plan was anticipated to preserve any genetic susceptibility towards polycythemia exhibited in, perhaps, less than all 4 parents. It might also enable one to determine if a particular parental line was more influential on the development of polycythemia. The genetic makeup of the progeny is depicted in Table III.

c. Pathology and clinical chemistry

Polycythemic rats, randomly chosen experimental controls, and moribund or tumor bearing rats were terminated by $CO_2$ asphyxiation and complete necropsies were performed. A gross and microscopic examination was made of all major organs including lung, heart, liver, spleen, kidney, adrenal, intestine, stomach, urinary bladder and brain. Sections of spinal cord, muscle and tumor tissue were also collected. Weights of the heart, spleen, liver, and kidneys were recorded. All tissues were fixed in 10% buffered formalin, processed by standard microtechniques, and stained with Harris' hematoxylin and eosin prior to examination by light microscopy. Serum and urine were collected from polycythemic rats, their nonpolycythemic littermates,

agreed to collaborate on these studies and conduct selected, specialized tests which his laboratory routinely performs. Red cell mass was determined by labeling an aliquot of autogenous RBCs with a known amount of $^{51}Cr$, infusing the labeled RBCs via the tail vein, and measuring the radioactivity in a volume of RBCs after 30 minutes. The blood volume was calculated with the Hct value.

Erythropoietin levels were to be determined by a RIA technique, but this analysis was delayed and inconclusive. An alternate method was used whereby Dr. Gerald Krystal, B.C. Cancer Research Centre, Vancouver, B.C. [89] determined Ep levels using a microassay based on $^{3}H$-thymidine incorporation into spleen cells from mice treated with phenylhydrazine. Phenylhydrazine increases by 10 fold the number of splenic cells of which over 90% are erythroid. These spleen cells incorporate the labeled base in proportion to the amount of Ep in a serum sample. Sensitivity is as low as 2 mU Ep/ml serum.

Cell culture characteristics of erythroid precursor cells from femoral bone marrow was determined by Dr. Adamson's group. Cell growth response from sacrificed polycythemic rats was compared to controls. Normal erythroid precursors require Ep for BFU-E and CFU-E formation in culture. In autonomous erythroid differentiation, CFU-E cultures form without addition of exogenous Ep.

Complete blood counts were performed on polycythemic and control rats. Values determined with a Coulter counter, Model ZBI, included counts of RBCs, white cells, and platelets, Hb concentration, Hct, and MCV. Blood smears were made on glass slides, stained, and examined

tributed 4 per cage into hanging wire cages for the remainder of the experiments.

2. Characterization of Polycythemia

All rats were observed daily for gross signs of morbidity including rough appearance of the hair coat, dehydration, cyanosis or plethora, dyspnea, diarrhea, abnormal nasal and eye discharges, unusual motor activity, alertness and gross tumor development. Body weights of progeny were recorded at 1, 2 and 4 months of age.

a. Hematology

Packed cell volumes (Hct) were determined weekly for offspring from groups 2, 3 and 8 (Table II) since these rats were primary suspects for developing polycythemia as reported in the original study by Koller et al, 1979. One fourth of the progeny from each of the remaining 5 groups were also tested weekly for % Hct. This sampling method allowed all rats to be bled at least monthly. Tails were pricked with a sterile lancet, and blood was collected in a microhematocrit tube, sealed, and centrifuged to to give the Hct reading. As cyanotic or polycythemic rats were detected in the secondary suspect groups, the measuring of Hcts was increased to a weekly basis for each litter having a sibling with a Hct > 50%. Polycythemic rats were diagnosed when the Hct exceeded 60%.

Polycythemic rats were initially paired up with age, weight and sex matched controls and rushed to the University of Washington School of Medicine to determine red cell mass, Ep levels, and cell culture characteristics. Dr. John W. Adamson, Department of Hematology,

Fifty three (53) weanling female Wistar rats from a closed colony were divided into 8 qroups as depicted in Table II. One group was to serve as negative controls, 5 groups were designed to test the interrelationship of the three chemicals on the production of polycythemia, and 2 groups were included to conduct a cursory dose-response test of MM (group 3) and ENU (group 8). The dams were fed a diet containing 0 or 10 ppm MMCys for about 13 weeks from time of weaning through gestation. One male Wistar rat was placed into a cage with 3 females that had been treated for 10 weeks. Males that would not breed within 1 week were replaced with a male littermate. Vaginal smears were obtained daily from each female rat by rinsing the vaginal opening with a sterile eyedropper containing sterile saline. The vaginal wash was smeared onto a microscope slide, dried, stained with 0.4% methylene blue and examined under a light microscope. The day of conception was determined by the presence of sperm or a vaginal plug in the vaginal wash. Pregnant females were placed in polycarbonate cages to litter.

Six of the 8 groups of dams were gavaged with varying doses of EU and $NO_2$ on days 17, 18 and 19 of gestation. All 53 dams were weighed on day 17 and gavaged with approximately 3 ml total of either the chemical solution or physiological saline. Pregnant females were observed twice daily following day 19 of gestation until parturition. Newborn pups were counted and weighed and stillborn pups were removed from the cages. All pups were weaned at 3 weeks of age and dis-

desired in a kg of powdered rat chow. A batch of 400g of premix would prepare 20 kg of chow with 10ppm MMC. The premix and chow were thoroughly blended in a stainless steel bowl by a large commercial mixer. All other mercury analogs were prepared in a similar manner. Enough mercurial feed was prepared to last the entire experiment.

The nitroso compounds were usually administered as the alkylurea and sodium nitrite precursors. Solutions of EU and $NO_2$ were freshly prepared each day that dams were gavaged. To repeat the original study, 50 mg/kg of EU and 25 mg/kg $NaNO_2$ were administered once a day for 3 days. Based on a dam's weight of 300g, she would thus receive 15mg (.17mM) EU and 7.5 mg (.11mM) $NO_2$. To simplify dosir based on weight, stock solutions of EU were made at 10 mg/ml and $NO_2$ made at 5 mg/ml. A 300 g rat would be gavaged with 1.5 ml of each solution for a total volume of 3.0 ml. For each 20 g of a dam's weight from 300 g, the dose was adjusted by 0.1 ml EU and 0.2 ml $NO_2$.

Later studies used equimolar (.17 mM) amounts of precursors so that a 300 g pregnant dam received 15g EU and 12g $NO_2$. The pH of the precursor solutions were adjusted to 3.0 to optimize formation of ENU in the rat stomach. Other nitroso analogs were prepared to also give a daily dose of 0.17 mM to dams. Treated mice received ENU precursors in the diet, rather than by gavage, during the third trimester as 1.0 g/l $NO_2$ in drinking water and 0.32% EU in the feed.

E. Experimental Designs

1. Attempt to Reporoduce Original Study

within a range of 68-73°F. Water bottles, cages and feed trays were sanitized at least twice weekly.

D. Preparation of Mercurial and N-Nitroso Compounds

To improve the solubility of mercury compounds used in this study, the chloride salts of MM, phenyl mercury and ethyl mercury were chemically reacted with L-cysteine [122]. The organomercury-cysteine compounds are less hazardous to workers and are more stable and easier to mix into powdered feed. Methylmercury chloride in aqueous solution was purchased in 250 ml quantities of 1000 ppm MMC, equivalent to 1 mM or 250 mg MMC per bottle.

A 5% molar excess of cysteine (M.W. = 121.6 g/M) solution was prepared by stirring 0.128 g cysteine in 50 ml distilled water and adjusting the pH to 7.0. The cysteine solution was slowly dripped into the stirring MMC solution to give a total of 300 ml MM-cysteine (MMCys) solution. One hundred (100) g of dry milk was dissolved into the MMCys solution, and the milk was frozen onto the sides of Virtis glass jars by means of a dry ice-acetone bath. The milk with MMCys was lyophylized (Virtis Freezemobile 12, Gardner, N.Y.) at -70°C and 10 mTorr until dry. The lyophilized milk was finely ground with a mortor and pestle, sifted, and weighed in a plastic gallon jar.

A small sample of the MMCys-containing milk was analyzed for mercury to calculate the yield, usually 80% or about 200 mg equivalent of MMC. Caseinate was added to the milk to give a 400g premix containing 1 mg MMC per 2 g premix. After vigorous mixing of the dry milk and casein, 2 g of the premix were added for each 1ppm MMC

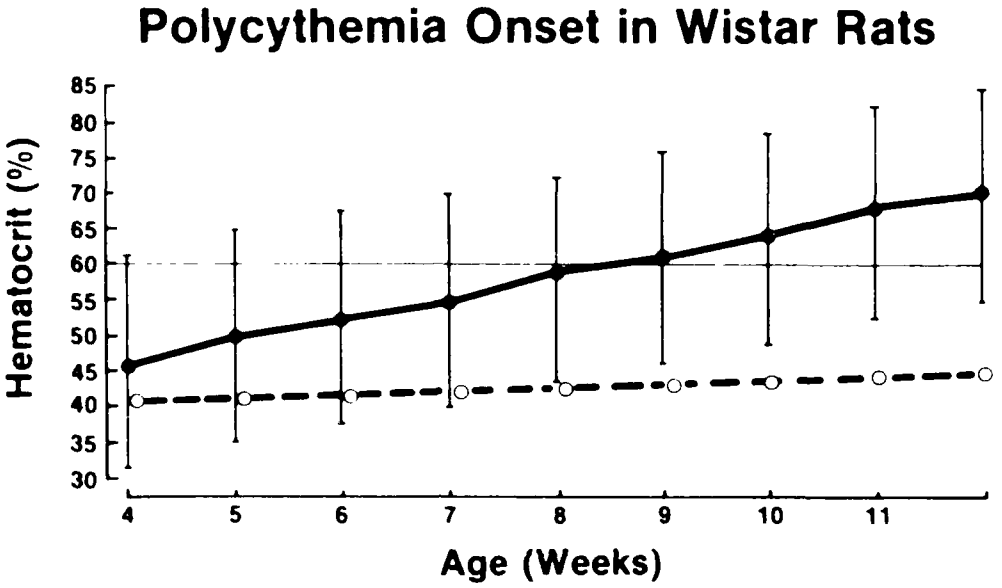


Figure 5.

The mean onset of polycythemia in 18 rats was approximately 8 weeks of age for rats that developed polycythemic by 12 weeks of age. Polycythemia was diagnosed when the hematocrit (Hct) exceeded 60%. The Hcts ranged from 60 to 83% for polycythemic rats, and 36 to 52% for control rats. The polycythemic rat Hcts became significantly different ($P < .05$) from controls at 5 weeks of age.

diagnose polycythemia, 9 additional offspring developed Hcts of 55 to 60%. Polycythemia was not observed in dams, control rats, or rats exposed only to EU and $NO_2$ without MM. Surprisingly, the progeny from group 3 did not develop polycythemia after their mothers were treated with MM for a shorter duration than the dams from group 2.

B. Characterization and Pathogenicity Studies

1. Lineage and Toxicity in Dams

All litters exposed to MM, EU and $NO_2$ (groups 2 and 8) had rats that developed polycythemia (Table VI). Groups 4, 5 and 6 had rats that were treated with MM alone or with MM and either $NO_2$ or EU, and polycythemia developed in 60%, 50% and 25% respectively of these exposed litters. Within the 11 litters from 5 groups with polycythemic rats, 47% of the polycythemic animals were males while 28% were females (Table VII). No difference in the incidence of polycythemia by gender was observed between the 5 polycythemic genotypes or between the 5 treatment groups (Table VII). The results do not indicate a clear genotypic influence on phenotypes, except that representatives from all 5 genotypes in MM-treated groups developed polycythemia.

Dams generally showed minor signs of chemical toxicity resulting from MM treatment. Occasional ataxia, crossing of rear limbs, and rough hair coats were observed. The major toxic effect of chemical treatment was on the reproductive capacity of dams (Table VIII). Percent litter rate was reduced to 46% in group 2 rats compared to an 83% or higher successful conception rate in all other groups. Only 4

of 6 litters (67%) from group 2 had pups survive to weaning at 3 weeks of age, compared to 80% or higher for all other groups. Numbers of pups born alive per litter was only significantly ($P \leq .05$) reduced in group 5 compared to the controls (group 1). Survival at weaning was less than half that of controls (94%) for group 2 progeny (39%), and was also reduced in groups 4 and 6 progeny (58 and 59%). Survival was slightly reduced to 84% for offspring from group 3 and 5, but about equal to controls for groups 7 and 8.

2. Clinical Signs and Weights

Most rats with early onset of polycythemia appeared small, weak and lethargic. As the Hct rose above 60%, the rats became more cyanotic, dyspneic, and depressed. Some would collapse upon handling. Clinical dehydration, as judged by pinched skin and position of eyes in their sockets, was rarely observed. Polycythemic rats with delayed onset showed similar signs, except they did not appear smaller than littermates and they often had an enlarged thorax. As the rats approached one year of age, occasional ataxia, rear paralysis, or head-tilt and circling would appear randomly in all treated groups of rats.

Body weights for all male and female offspring are shown by groups in Table IX. At 1 month of age, rats from group 6 were generally heavier while rats from groups 7 and 8 were lighter than controls. At 2 mo of age, males from group 2 weighed significantly less than male rats from all other groups. Group 8 females had the lowest average weights. At 4 mo of age, the control males weighed

significantly more than males in all other groups, and male rats from groups 2, 5 and 8 had the lowest weights. Four month old female control rats weighed significantly more than females from groups 2, 5 and 7. At both 2 and 4 mo of age, rats from groups 2 and 8 had the lowest average weights of any groups.

Body weights of female polycythemic rats were significantly lower at 1 mo of age when compared to female littermates (Table X). Polycythemic females also weighed less at 2 mo and 4 mo compared to their littermates, and both these groups weighed less than controls at 2 and 4 mo of age. Male polycythemic rats weighed significantly less than male rats from the control group at all ages measured, and less than male littermates 2 mo of age. At 4 mo of age polycythemic rats averaged less in weight compared to controls.

3. Hematology

The Hcts, RBC counts, Hb concentrations, and WBC counts in polycythemic rats were all elevated significantly ($P \leq .05$) higher than in control rats, except for the more than doubled WBC count where $P = .073$ (Table XI). Mean platelet counts tended to be reduced ($P \leq .10$) in polycythemic rats, but two polycythemic rats had counts exceeding $1 \times 10^6/\mu l$ (data not shown).

Red cell masses were nearly doubled in polycythemic rats compared to controls ($P \leq .0002$) (Table XII). Erythropoietin (Ep) levels were elevated above control values in 4 polycythemic rats tested (Table XIII). Two polycythemic rats had Ep levels approaching 2000 mU/ml. Phlebotomy caused the Ep levels in serum to increase 2

1/2 times (1800 to 4800 mU/ml) one week later in a rat with polycythemia induced by BNU. Control levels of Ep ranged from 10 to 37 mU/ml. $O_2$ saturation of blood obtained by cardiac puncture was analyzed in 2 control and 2 polycythemic rats; however, the polycythemic rats had advanced polycythemic disease (Hcts>70%) and hypercapnia. The $PO_2$ and $PCO_2$ levels in these animals averaged 16 and 91 mm Hg compared to 74 and 42 mm Hg in controls.

Differential counts of leukocytes were similar in polycythemic and control rats and consisted of approximately 70% lymphocytes, 25% heterophils, and 5% monocytes. Diseased rats with leukocytosis often had a left shift, or increase in band cells. Red and white cells typically had normal morphology with slight polychromasia of RBCs. Reticulocytes averaged about 1 to 2% of RBCs but increased to around 10% with 1% nucleated RBCs in the few anemic rats. A few polycythemic blood samples were tested for RBC indices which were similar to controls.

No significant differences were observed in numbers of erythroid precursors; however, total splenocytes and CFU-GM and CFU-M were greatly reduced in the hematopoietic tissues from polycythemic rats (Table XIV). Another experiment conducted by Dr. Adamson, Seattle, WA, [5] revealed that the erythroid stem cells were Ep-dependent in cell culture and responded similarly to Ep as did stem cells from control rats.

4. Gross, Microscopic and Clinical Pathology

Gross pathology. Stillborn pups from all litters exposed to MM had occasional (11%) limb malformations or absence of hind limbs and the tail. Three offspring from ENU-treated groups had hydrocephaly and 2 had microopthalmia. Only 14 (2%) of more than 700 progeny from all experiments developed tumors before one year of age. Tumor incidence was not corrected for early deaths or euthanasia for sampling. Twelve progeny (11 Wistar, 1 SD) developed focal spinal cord hemorrhage, malacia, and tumors near the thoracolumbar junction. Rats with spinal cord tumors had rear ataxia or paralysis, soiled perineal areas, scoliosis, and emaciation. Two progeny that were exposed transplacentally to phenylmercury developed lymphocytic lymphosarcomas which infiltrated and caused enlargement of the spleen and liver, and spread to small foci in the lungs and kidney. One dam from group 8 (Table II) also developed a spinal cord tumor at 9 mo of age. Mammary gland tumors were detected in the subaxillary area of an 18 mo old dam from group 4, and in 2 untreated dams over 2 years old that were used as breeding stock.

Skin of polycythemic rats was dry and dark-purple on the feet, ears and tail. Early onset rats weighed 25% less than littermates at weaning, while late onset rats were similar in size to littermates but were 'barrel-cnested'. At necropsy most internal organs were enlarged and engorged with thick, dark blood. Percent body weights of the heart and spleen were significantly increased in polycythemic rats (Table XV). The bone marrow was dark red and abundant in polycythemic

rats. The lungs were normal in rats under 1 year of age, but there were areas of redness and firmness in some older non-polycythemic rats with pneumonia. Pneumonia was only observed in breeding stock or treated dams (Table II) over 18 mo of age that were necropsied to determine causes of dyspnea.

Histopathology. Except for aged breeding animals with pneumonia or the above rats with tumors, few microscopic lesions were observed. Tissue sections from sampled control and polycythemic rats occasionally revealed mild peribronchiolar lymphoid hyperplasia. A mammary cystic pappillary adenoma, mixed mammary tumor, and a fibroadenoma were diagnosed in the aged dams. Six of the 12 spinal cord tumors were mixed gliomas with a prevalance of oliogodendrocytes and anaplastic glial cells. Neural tissue hemorrhage and necrosis was observed in sections from the remaining spinal cord tumors, but no neoplastic cells were present probably due to improper tissue sampling.

The white matter from brains of dams exposed chronically to MM had occasional mild neuronal degeneration and vacuolation. The livers from polycythemic rats had mild centrolobular degeneration, and the vascular channels were enlarged and congested. Numerous cortical cysts of various sizes occurred in the kidneys of offspring in all MM-exposed groups. Cross sections of femurs from polycythemic rats had a significant increase in cellularity and a possible ($P \leq .10$) decrease in numbers of megakarocytes compared to control femoral sections (Table XVI).

Clinical pathology. Cursory urinary analyses using clinitest lab sticks showed no major differences between pH, protein, blood, glucose, or urobilonogen levels of urine from polycythemia and control rats. Serum chemistry tests showed a significant ($P \leq .05$) increase of mean BUN levels in male rats, an increase in alkaline phosphatase, and hypoglycemia in polycythemic rats (Table XVII). Serology was performed on 5 randomly chosen treated and untreated Wistar rats, and all 5 had positive titres for Mycoplasma pulmonis as determined by an ELISA test at the Laboratory Animal Resource Center, Washington State University. All were negative, however, for viral titres to Sendai virus, pneumonia virus of mice (PVM), Kilham rat virus (KRV), H-1 parvovirus, mouse hepatitis virus (MHV), and sialodacryoadenitis virus (SDA).

5. Dose and Duration of Exposure Effects

Weanling rats exposed to increasing levels of 4 to 24 ppm MM in the diet for 13 weeks were gavaged with ENU precursors during the last trimester of gestation. Of 29 experimental pups that were weaned, only 1 female from the 10 ppm group developed polycythemia (Hct = 63%), at 4 months of age. Clinical toxicity in the dams was dose-related (Table XVIII). Only 5 dams from the lower exposure groups (4 to 12 ppm) produced litters.

In another experiment weanling Wistar rats were exposed to 10 ppm MM for varying durations and gavaged with .17 mM of ENU for 3 days during the third trimester of gestation. Dams were exposed to MM for 6,7,8,9, or 10 weeks prior to parturition, and 23,22,15, 13 and 14

pups were weaned respectively per weeks of MM treatment. No polycythemia nor other disease was observed in the progeny from birth to 10 months of age. In the experiment to reproduce the polycythemia (Table II), the infertile dams and fertile dams from group 2 that had weaned their pups (Table VIII) were again placed on a 10 ppm MM diet at about 16 to 17 weeks after initially placed on the MM diet as weanlings for 13 weeks. After 1 week of renewed exposure to MM, the dams were mated and gavaged with ENU precursors on days 17, 18 and 19 of gestation. Exposure to MM continued until parturition. None of the offspring from the resulting 5 litters developed polycythemia. One of these pups was analyzed for Hg content which was about 25% lower (2.2ppm) than levels in pups from dams fed 10ppm MM for only 13 weeks (Table XIX).

Tissue levels of mercury. Mercury averaged about 18 ppm in day-old pups from groups 2 and 4 (Table XIX). Levels of mercury in blood from control rats were low at .08 ppm. Two 9 mo old progeny that were littermates of polycythemic rats had 12.3 and 71.5 ppm mercury in blood. The dam fed 20 ppm MM had higher levels of mercury in all tissues analyzed than did the dam fed 10 ppm MM. Hair to blood ratios of mercury in the MM-treated dams was about 10 to 1. Ethylmercury at 10 ppm in the diet produced about 20% of the blood level of mercury compared to the MM-treated dam. Blood levels of mercury from a dam fed 10 ppm phenylmercury were about equal to non-treated control levels.

Disease transmission. In separate experiments to investigate possible horizontal or vertical transmissions of the polycythemic

disease, no polycythemia was observed in offspring or in blood recipients up to 12 months of age.

6. Immunological responses

Immune functions of nontreated control rats and transplacentally exposed rats did not greatly differ in the categories tested. Humoral immunity was significantly ($P \leq .05$) lower at the 1:4000 serum dilution (Table XX). Organ weights were recorded for these 20 week old rats, and treated females had a significantly increased percent kidney weight compared to males and nontreated females (data not shown).

Skin grafts were transplanted between 3, six week old male Wistar rats (nontreated). One graft survived for 1 month when the experiment was discontinued. Two grafts were rejected between 7 and 10 days after transplantation. Rejected grafts became discolored, shrunken and dried.

C. Induction by Chemical Analogs

Mercury analogs. Nine dams were divided equally into 3 groups and fed 10 ppm of ethylmercury (EM), phenylmercury (PM) or mercuric chloride ($HgCl_2$), all conjugated with cysteine. After 10 wks the dams were mated and gavaged with ENU precursors during the third trimester. Eight litters were born, but only 5 litters of animals survived to weaning. Four EM progeny, 17 PM progeny and 21 $HgCl_2$ progeny were bled and observed regularly for 9 months without any detection of polycythemia.

Nitroso analogs. Seven dams were fed 10 ppm MM for 10 weeks prior to breeding. During days 17, 18 and 19 of the third trimester,

2 dams received .17mM diethylnitrosamine, 2 dams received .17 mM of butylurea and sodium nitrite, and 3 dams were gavaged with .17 mM of ethylurea and sodium nitrite. Thirteen ENU pups, 13 BNU pups, and 6 DEN pups were weaned. Two rats exposed to BNU precursors developed polycythemia that appeared identical to previous cases. Onset was at 8 months. One rat exposed to ENU precursors also developed polycythemia at 10 months of age.

D. Susceptibility of Alternate Species

All 4 SD dams, 1 of 4 Lewis dams and 3 of 4 Wistar dams produced litters following 13 weeks of dietary exposure to 10 ppm MM and gavage with ENU precursors during the third trimester. Six litters of similarly treated Swiss-Webster mice were also obtained. Hematocrits have been followed for 4 months without onset of polycythemia in any of the groups. However, all three strains of rats had a transient rise of Hcts in several individuals between 3 and 4 months of age. The elevated Hcts ranged from 50 to 58% and lasted for 2 to 6 weeks. Three of 5 Lewis rats (all males), 5 of 29 Wistar rats (all males), and 25 of 42 SD rats (18 males) developed transient, mild erythrocytosis. No cases have exceeded a Hct of 60%, and most rats had normal hematocrits at 6 mo of age. Only mild cyanosis was observed in rats with Hcts approaching 60%. Fifteen rats continued to have a Hct in the mid 50% range at 6 months of age. Exposed mice have not developed any increase in their hematocrits.

## V. DISCUSSION

Repeated study. The first objective of this research was met by repeating the original study and reproducing polycythemia in 42% of progeny from dams exposed to 10 ppm MM, 50 mg/kg EU and 25 mg/kg $NO_2$ (Table V). The original study detected a 41% incidence of polycythemia in similarly treated rats [81,85]. First onset was at 4 weeks of age and most cases developed between 2 and 3 months in the original report [81]. The first case in this study appeared at 6 weeks of age, and the mean onset was 8 weeks of age for progeny that developed polycythemia by 12 wks of age (Figure 5). Methylmercury-cysteine was administered to rats in this study whereas rats in the initial study were exposed to methylmercury-chloride dissolved in corn oil. Both MMCys and MMC are distributed similarly and undergo minimal biotransformation into inorganic mercury in rats [122].

Progeny from all groups of dams exposed to chronic MMCys developed polycythemia in the present study (Table V). Incidence of polycythemia for litters within groups and for rats within litters was highest in group 2 and 8 which received all 3 chemical treatments. Doubling the dose of EU and $NO_2$ in group 8 did not increase the incidence or affect the onset of polycythemia compared to group 2 rats. Polycythemia did not develop in group 7 progeny exposed only to EU and $NO_2$, nor in group 3 offspring from dams exposed to MMCys for 4 weeks. These results suggest that MM is the essential prenatal

chemical, and that chronic treatment of dams is required to induce polycythemia in Wistar rats.

The higher incidence in male rats compared to females is a similar gender-related feature of human PV [175]. Early age of onset, regression of erythrocytosis in up to 30% of the rats and development of anemia in several rats are uncharacteristic of PV in humans [2,5,175].

The clinical signs of polycythemia were similar in the original report and in the repeated study. The hemograms of polycythemic rats also showed a similar elevation in RBC and WBC counts and in Hcts (Table XI). In addition, the Hb concentration was significantly elevated which is characteristic of most polycythemias [55]. Several polycythemic rats experienced leukocytosis and thrombocytosis, which are features of human PV [4,175]. As previously observed, the percent body weights of the spleen and heart, but not the liver and kidney, were significantly higher in polycythemic rats (Table XV). Bone marrow was hypercellular in polycythemic rats from both studies (Table XVI). Because the present studies were not designed to observe lifetime effects of treatments, only a small incidence of neoplasia was observed in progeny up to one year of age. Tumor latency varied with dosage and ranged from 377 to 530 days in groups which produced polycythemic rats during the original study [120].

Fertility and reproductive performance were similar in group 4a from the former study and group 2 of the present study (Table VIII) [119]. Percent litter rate and litter sizes were lower while percent

stillborn pups was higher in groups treated with chronic MM, EU and $NO_2$ in both studies. Birth weights were significantly reduced from controls in all treatment groups in the present study, and also for the polycythemia rat groups in the original study. Survival at weaning was 21% previously and 39% presently for group 2 progeny. Previously, reproductive performance deteriorated with increased dose of EU and $NO_2$, but this dose response effect appeared reversed in groups 2 and 8 in the present study (Table VIII). Group 4e in the original study had identical exposure as group 2 in the repeated study, but litter size, % stillborn, and birth weights were not different from controls for group 4e rats [119].

Tissue levels of mercury in maternal kidneys of dams fed 10ppm MMC in the original study were 63.3 ± 23 ppm compared to 80 ppm in a dam fed 10 ppm MMcys and 135 ppm in a dam fed 20 ppm MMcys during the present studies (Table XIX) [119]. Pups exposed to MM only or to all 3 chemicals had 11.3 ± 2.4 ppm Hg or 19.8 ± 4.1 ppm Hg in the former study, and 16.8 ± 1.8 ppm Hg or 19.5 ± 1.5 ppm Hg in the current study.

Characterization. Transplacental exposures of Wistar rats to all three chemicals produced polycythemia in 100% of the litters weaned (Table VI). Although chronic MM treatment of dams alone or with a single ENU precursor could induce polycythemia, incidence was higher in progeny exposed to all three chemicals via the placenta. Since the halflife of MM in rats is about 20 days and steady-state levels are established at about 30 days following a dose of 1 mg MMCys/rat/week,

AD-A156 938 CHEMICAL-INDUCED ERYTHROCYTOSIS IN WISTAR RATS: ASSESSMENT AS A MODEL FOR..(U) AIR FORCE INST OF TECH WRIGHT-PATTERSON AFB OH G M HENNINGSEN MAY 85 2/2

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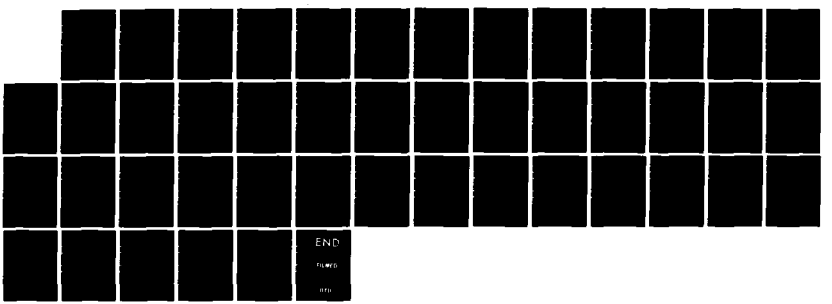

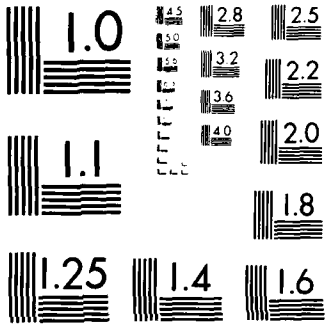

it seems unusual that progeny from group 3 dams did not develop polycythemia (Table V) [122]. Perhaps chronic adverse effects in dams from MM exposure are required in addition to certain steady-state levels of MM in pups to induce toxic changes that lead to polycythemia in some rats.

Pedigrees of polycythemic rats were traced to determine a pattern of inheritance [53]. Dominant alleles may have complete or intermediate expression, produce traits in about half of the offspring, and result in individuals without a trait being unable to transmit the trait to offspring. Recessive alleles produce traits only in homozygous individuals with first appearance only in siblings but not parents. An average of 1/4 of the siblings are affected with equal sexual distribution unless a recessive gene is sex-linked. Sex-linked recessive traits occur much more frequently in males than females, are passed through daughters to half their sons, and never pass directly from father to son. Over 20 sex-linked genes have been reported in mice but none in extensively studied rats [53].

Results showed that 12 of 19 (63%) litters exposed to MM developed polycythemia (Table VII). The ratios of polycythemic to normal phenotypes for rats from all 5 genotypes averaged about 1:3 (Table VII). Of the progeny from 19 litters in groups 2, 4, 5, 6 and 8, about 41% were males and 20% were females (Tables V, VI). The ratio of polycythemic to non-polycythemic phenotypes most closely represents a sex-linked recessive allele. However, the polycythemia could be expressed by an intermediate-dominant allele since the ratio

of rats with Hcts greater than 50% to less than 49% followed a 1:1 ratio of expression [53]. No parental-lineage influence on phenotypes was detected (Table III).

One of the earliest signs of MM toxicity is anorexia which can reduce body weights [111]. Weaning weights were not affected greatly from controls except for group 7 and 8 rats which weighed less (Table IX). The generally lower body weights observed for rats from groups 2, 5 and 8 corresponded with the higher incidences of polycythemia in these groups (Table V). Polycythemic rats weighed less than non-polycythemic littermates and control rats at all ages measured (Table X); whereas polycythemic littermates weighed less than controls only at 2 and 4 mo of age.

Diagnosis. Nearly doubled red cell mass values indicated absolute erythrocytosis (Table XII). Clinical dehydration was seldom observed, and similar total protein/albumin levels in control and polycythemic rats support the conclusion of normal hydration (Table XVII). Serum Ep levels were elevated in all polycythemic rats which is diagnostic for secondary erythrocytosis (Table XIII). No secondary causes of polycythemia were observed by gross and microscopic examinations of tissues from the previous or present study. Oxygen saturation was extremely lowered in 2 rats with advanced polycythemia (Hcts $\geq$ 73%), and a lowered $SaO_2$ level would be expected from the extreme hyperviscosity [5]. The $SaO_2$ analyses need to be repeated on acutely polycythemic rats. The $SaO_2$ values, however, are unlikely to be low since earlier studies found 2,3-DPG levels to be similar in

control and polycythemic rats [85]. This enzyme is usually elevated in RBCs from hypoxic patients with low $SaO_2$ values [68].

Unless these Wistar rats produce genetically-controlled excess amounts of Ep, this disease can most likely be diagnosed as secondary-appropriate polycythemia (Figure 1). Since polycythemic rats had tachypnea and no evident cardiopulmonary lesions, the most likely cause would be abnormal $O_2$ unloading. Renal cysts were observed in rats from all MM-treated groups, and no relationship between cyst size or numbers could be linked to polycythemic rats. Abnormally high $O_2$ affinity of erythrocytes had previously been found in polycythemic rat blood, but its significance was uncertain (Figure 4) [81,84].

Toxicopathological findings. Hematology results showed occasional leukocytosis, often with left shifts, and thrombocytosis in some polycythemic rats. These features, along with splenomegaly and occasional cardiomegaly and hepatomegaly, are typically diagnosed in human PV patients. Elevated Ep levels rule out this rat disease as PV, and heightened Ep levels following phlebotomy supports an appropriate cause of polycythemia (Table I) [2,88,115]. Bone marrow cell culture studies showed that polycythemic rat erythroid stem cells were Ep-dependent as were controls [5]. The reduction in CFU-GM and CFU-M from polycythemic rats could possibly be due to a shift towards erythroid stem cell production at the expense of leukocyte precursors (Table XIV) [175].

Both MM and ENU are potent teratogens in animals [24,71], and several gross malformations were observed in stillborn pups. Both

chemicals are carcinogens; however, MM is weak and ENU is a strong neoplastic agent - especially in utero [37,119]. Spinal cord tumors were most often encountered in the repeated study. The enlargement of internal organs may be related to the hypervolemia and hyperviscosity of blood in the polycythemic rats whose Hct exceeds 60% (Table XV). Anoxia due to reduced circulation of viscous blood could account for the mild centrolobular hepatic degeneration seen in some livers from polycythemic rats.

Rats commonly develop renal cysts following perinatal MM exposure [111]. Only a fraction of rabbits with experimentally induced cysts developed increased Ep production and erythrocytosis [6]. Intracyst pressure had to be between 230 and 330 mm $H_2O$ to cause an increase in Ep production due to ischemia [6]. Since all MM treated Wistar rats had varying sizes and numbers of renal cysts, their direct role in the production of erythrocytosis is probably minor.

Unlike many human PV cases, polycythemic rats tended to have reduced numbers of megakaryocytes in bone marrow (Table XVI); however, like human PV, the marrow was hypercellular [17]. The only clinical pathological differences between polycythemic and control rats were a slight elevation in alkaline phosphatase and lower blood sugar levels for diseased rats (Table XVII). The blood sugar levels were within normal ranges for Wistar rats [142], and the alkaline phosphatase elevations were probably due to hepatic congestion and centrobular hypoxia which result in degeneration and leakage of enzymes from hepatocytes [40]. The mild but significant increase in BUN in serum

from male rats could be due to increased protein-catabolism or a decrease in glomerular filtration rates [40]. Serological tests detected Mycoplasma pulmonis titers in all non-polycythemic Wistar rats tested. No significant lesions were detected in the lungs of any polycythemic rats which could account for compromised respiratory function.

Dose response studies were conducted to try and increase the frequency of polycythemia and decrease the induction time, but the experiments were generally unsuccessful. A possible explanation for lack of success is the use of non-fresh $NaNO_2$ to gavage the dams in the later studies. The melting point of the $NaNO_2$ was elevated above 271°C for pure $NaNO_2$, to 284°C. Oxidation of $NaNO_2$ produces $NaNO_3$ which melts at 308°C. Assuming linearity of the melting point for a mixture of $NaNO_2$ and $NaNO_3$, the yield of $NaNO_2$ was only 67%.

Polycythemia did develop in progeny exposed to the leukemogenic butylnitrosourea but not in progeny exposed to diethylnitrosamine. Progeny did not develop polycythemia following ethylmercury, phenylmercury nor mercuric mercury exposure for possibly the same reasons above regarding non-fresh $NaNO_2$. The duration-response study examined progeny exposed to .17mM ENU and derived from dams treated with 10ppm MMCys for varying lengths of time. The lack of polycythemia in these rats could be partly due to insufficient exposure as seen between groups 2 and 3 in the repeated study (Table V). Other unidentified genetic or environmental and experimental factors might also be involved in the lack of polycythemia in these offspring. Repro-

ductivity was low in all the dose-response and analog studies which may also partly account for the occurance of only 4 polycythemic rats in the latter experiments.

Tissue levels of MM in blood from littermates of polycythemic rats appeared quite high 9 mo after transplacental and lactational exposure (Table XIX). Unfortunately, only 2 rats were sampled. While errors in sampling could possibly explain the 71.5 ppm level in one rat, studies have shown that equivalent exposure of MMCys in adult rats resulted in about 100ppm blood levels for several months after exposure [122]. Kidney levels of MM in Minamata disease victims persisted above 20ppm for over 2 years after MM exposure was essentially eliminated [76]. The lower amounts of mercury in tissues from ethylmercury and phenylmercury exposure were expected due to their faster carbon-mercury bond cleavage and elimination as inorganic mercury [62].

Polycythemic rats were not available when the multiple immune function assays could be performed, but Wistar rats treated as group 2 (Table II) were available to compare with non-treated control rats. Most immune functions were identical between the 2 groups except for a significant decrease in IgG production in the treated rats (Table XX). Polycythemic rats may possibly exhibit further alterations in immune functions. Skin grafts survived on hosts for at least one week in 2 rats and for a month in another rat. Although these Wistar rats are not inbred, the delayed graft rejection in 2 animals and acceptance in

a third could indicate this colony is closely related genetically [175].

Individual Wistar rats and the Lewis and Sprague-Dawley rats may express a milder hematological toxicity compared to polycythemic Wistar rats with Hcts $\geq$ 60%. In an earlier study with Fisher 344 rats [5] and presently with the Lewis, Wistar, and Sprague-Dawley rats, transplacental exposure has produced transient elevations of Hcts between 50 and 58% without other clinical signs of disease. Perhaps the polycythemic Wistar rats lack the ability to properly compensate for the hematological damage exerted by MM and ENU in utero.

Animal model. The purpose of this research project was to determine if the polycythemic rats indeed had primary polycythemia that would serve as a useful model for human PV [5,81]. Presently there is no abundant, economical and erythremic animal model of human PV with which to study this neoplastic disease [3,5,132]. The results of this research have shown that the polycythemic rats do not have PV, because Ep levels are elevated (Table XIII) and bone marrow stem cells are Ep-dependent [5].

This chemical-induced blood dyscrasia retains potential, however, for use as a model of secondary-appropriate polycythemia [149]. It also retains some features of clinical PV, including splenomegaly, occasional leukocytosis and thrombocytosis, marrow hyperplasia, and higher incidence in males.

Since no apparent secondary causes of polycythemia have been detected, the diagnostic algorithm (Figure 1) and previous $O_2$ affinity studies (Figure 4) would suggest that an increased RBC affinity for $O_2$ may be the cause of the erythrocytosis. Normally in man, high $O_2$ affinity hemoglobinopathies produce only mild elevations in Hcts [2]. However, cases of hemoglobinopathies have shown greatly elevated Hcts [14,175]. Some cases have also produced splenomegaly and leukocytosis [98,99]. Human hemoglobinopathies are usually inherited as a dominant trait [2,14], and over 250 Hb abnormalities have been detected in man [149]. The polycythemia in Wistar rats appears to be strain and colony specific, and it may be inherited as an intermediate-dominant trait or as a sex-linked recessive trait.

No cases of high $O_2$ affinity hemoglobinopathies have been detected in animals. Animal hemoglobinopathies are seldom reported and are considered extremely rare. Ethylnitrosourea has been shown to mutate Hb genes in mice [173]. Perinatal Hb genes are some of the most actively transcribed and exposed nucleophilic sites, and they may therefore be more vulnerable to MM and ENU [175]. Considering MM's extreme affinity for rat Hb and ENU's potent mutagenic properties, perhaps these chemicals act in concert to induce an alteration in Hb that may be expressed or repressed later in transplacentally exposed Wistar rats [34,139].

If the cause of erythrocytosis is a mutant high affinity Hb in polycythemic rats, the variability in the clinical course of the disease might be due to the amount of aberrant Hb within the rat

erythrocyte. Human polycythemia and thallasemia are known to vary in severity according to the amount of affected Hb within the patients' RBCs [14,152,173]. Intraerythrocytic enzymopathies could also explain the high $O_2$ affinity due to an altered Bohr effect or enzyme defect [65,68,149]. Hemolysate $O_2$ affinities must be determined to definitively diagnose a hemoglobinopathy-caused erythrocytosis [5,14]. Oxygen saturation levels should also be verified, but they are usually normal in all cases of hemoglobinopathies [14,17]. The implications of ususally small environmental exposures to methylmercury and N-nitroso compounds in utero is unknown for human hematological disorders or other diseases. Some nitroso compounds are potent leukemogens [125], and toxic synergism of MM and ENU has been observed in the fetus which is highly susceptible to damage by these chemicals [119,128]. Exposure to benzene and other chemicals has been implicated as the cause of some human polycythemia cases [138].

In conclusion, this research fulfilled the stated objectives by 1) reproducing polycythemia in Wistar rats, 2) characterizing the polycythemia as a secondary-appropriate type, 3) examining the pathogenesis of the disease to determine a probable cause for the polycythemia and 4) assessing the disease as a model for human polycythemia. If future tests confirm an increased $O_2$ affinity by Hb, this will be the first report of hemoglobinopathy-associated erythrocytosis in animals with which to model the more than 45 inherited cases in humans [173,175]. Furthermore, these polycythemic animals

may offer a valuable means to better understand the complex cellular and molecular events and factors that control hematopoiesis.

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TABLE I. CLASSIFICATION OF ERYTHROCYTOSIS

I. RELATIVE Erythrocytosis (decreased plasma volume)

- A. Hemoconcentration: burns, shock, diarrhea, acute high altitude, saline depletion, protein loss
- B. Stress (Giasböck's syndrome, splenic contraction in animals)

II. ABSOLUTE Erythrocytosis (increased red cell mass)

- A. Secondary (increased erythropoietin)
  - 1. Appropriate : hypoxia
    - a. $O_2$ loading defects : high altitude, cardiopulmonary disease, hypoventillation, carboxy/met/sulf-hemoglobinemias, congenital
    - b. $O_2$ unloading defects : high affinity mutant hemoglobins, ATP or DPG abnormalities, familial
    - c. $O_2$ transport to kidney : vascular stenosis, cysts, cobalt, hydronephrosis, transplants
  - 2. Inappropriate : autonomous
    - a. Neoplasims : kidney, liver, cerebellum, lung, uterus, ovary, adrenal, cobalt- or nickel-induced
    - B. Familial
- B. Primary (decreased erythropoietin)
  1. Polycythemia vera
  2. Benign
  3. Familial
  4. Other mylonroliferative disorders

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TABLE II. EXPERIMENTAL DESIGN TO PRODUCE POLYCYTHEMIA IN WISTAR RATS

| Treatment Groups | Exposures [a] MM (ppm) | EU (mg/kg) | $NO_2$ (mg/kg) | No. of Dams | Purpose [b] |
|---|---|---|---|---|---|
| 1 | 0 | 0 | 0 | 9 | Controls |
| 2 | 10 | 50 | 25 | 12 | Polycythemia |
| 3 | 10 | 50 | 25 | 6 | Gestational MM [c] |
| 4 | 10 | 0 | 0 | 6 | MM only |
| 5 | 10 | 0 | 25 | 6 | EU omitted |
| 6 | 10 | 50 | 0 | 6 | $NO_2$ omitted |
| 7 | 0 | 50 | 25 | 6 | ENU only [d] |
| 8 | 10 | 100 | 50 | 2 | High ENU + MM |

[a] Weanling female Wistar rats were fed 10 ppm methylmercury-cysteine (MM) for 13 weeks. These rats were mated after 10 weeks of MM exposure, and gavaged with ethylurea (EU) and/or sodium nitrite ($NO_2$) on days 17, 18 and 19 of gestation.

[b] Hematocrits and weights of offspring were measured weekly after weaning, and appropriate diagnostic tests were performed to characterize polycythemic rats derived from the various treatment groups listed.

[c] This group of dams was fed MM only during breeding and gestation for a total of 4 weeks to determine if the induction of polycythemia could be shortened compared to the original study as repeated by group 2.

[d] EU and $NO_2$ spontaneously combine in the rat stomach to form the carcinogen, ethylnitrosourea (ENU).

TABLE III. GENOTYPES AND DISTRIBUTION BY TREATMENT GROUP

| Genotype [a] | No. of Litters per Experimental Group [b] | | | | | | | | Percent Parental Genes [c] | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | M1 | M2 | F1 | F2 |
| AA | - | - | - | - | - | 1 | - | - | 50 | 25 | 0 | 25 |
| BB | 1 | - | - | 1 | - | - | - | - | 50 | 25 | 25 | 0 |
| CC | - | - | 2 | - | 1 | - | - | 2 | 25 | 37.5 | 12.5 | 25 |
| AB | - | - | - | 3 | 3 | 2 | 5 | - | 50 | 25 | 12.5 | 12.5 |
| AC | - | - | 2 | 2 | - | 3 | 1 | - | 37.5 | 31.25 | 6.25 | 25 |
| AD | 1 | 2 | - | - | - | - | - | - | 25 | 37.5 | 25 | 12.5 |
| AE | 2 | 2 | - | - | - | - | - | - | 25 | 37.5 | 0 | 37.5 |
| BC | 3 | 4 | 2 | - | 2 | - | - | - | 37.5 | 37.5 | 18.75 | 6.25 |
| BD | 1 | 2 | - | - | - | - | - | - | 25 | 37.5 | 37.5 | 0 |
| BE | 1 | 2 | - | - | - | - | - | - | 25 | 37.5 | 12.5 | 25 |

[a] The 53 dams and their mates, bred to produce the experimental offspring were comprised of 5 genotypes designated A, B, C, D, and E. The offspring possessed one of 10 possible genotypes designated by the double-alpha characters.

[b] See Table II for explanation of treatment groups. Each of the 10 genotypes comprising the 53 litters was distributed as shown between groups.

[c] Four parental genotypes from the original colony were traced, and percent of each was calculated for the experimental offsprings' genotype.

TABLE IV. ANTIGEN TREATMENT AND SCHEDULE FOR MULTIPLE IMMUNOASSAY ANALYSIS [a]

| Day | Procedure/Antigen (Assay) | Dosage | Route |
|---|---|---|---|
| 0 | Inject KLH (ELISA) | 1 mg | sc (back) |
| | Inject BSA + FCA (DTH) | 100 mg | sc (base of tail) |
| 7 | Inject BSA (DTH) (2% heat-aggregated solution) | 100 ml | sc (footpad) |
| 8 [b] | Measure Footpad Swelling (DTH) | | |
| | Inject KLH (ELISA) | 1 mg | sc (back) |
| 14 [c] | Collect blood sample (ELISA) | | |
| | Collect Peritoneal Macrophages (PGE) | | |
| | Collect Spleen Cells (NK Analysis, IL2) | | |
| | Collect Tissue Specimens for Histopathology | | |

[a] KLH = keyhole limpet hemocyanin; BSA = bovine serum albumin; FCA = Freunds complete adjuvant; DTH = delayed-type hypersensitivity; PGE = prostaglandin $E_2$; IL2 = interleukin 2; NK = natural killer; ELISA = enzyme-linked immunosorbent assay.

[b] Rats were briefly immobilized with $CO_2$ to facilitate measurements.

[c] Rats were humanely euthanized by $CO_2$ asphyxiation.

TABLE V. INCIDENCE OF POLYCYTHEMIA IN WISTAR RATS EXPOSED TRANSPLACENTALLY TO METHYLMERCURY, ETHYLUREA AND SODIUM NITRITE

| Group | Exposures [a] MM | EU | $NO_2$ | No. Polycythemic [b] Total | M | F | No. Weaned Total | M | F | % Polycythemic Total | M | F |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | - | - | - | 0 | 0 | 0 | 77 | 42 | 35 | 0 | 0 | 0 |
| 2 | + | + | + | 10 | 6 | 4 | 24 | 10 | 14 | 42 | 60 | 29 |
| 3 | + | + | + | 0 | 0 | 0 | 54 | 31 | 23 | 0 | 0 | 0 |
| 4 | + | - | - | 3 | 1 | 2 | 37 | 9 | 28 | 9 | 11 | 8 |
| 5 | + | - | + | 5 | 4 | 1 | 22 | 11 | 11 | 23 | 36 | 9 |
| 6 | + | + | - | 2 | 1 | 1 | 36 | 19 | 17 | 6 | 5 | 6 |
| 7 | - | + | + | 0 | 0 | 0 | 61 | 27 | 34 | 0 | 0 | 0 |
| 8 | + | ++ | ++ | 7 | 4 | 3 | 21 | 13 | 8 | 33 | 31 | 38 |

[a] See Table II for exposure regimens. Group 3 rats were offspring of dams exposed to MM only during gestation. Group 8 rats were exposed to double the dose of EU and $NO_2$ compared to group 2 rats.

[b] Polycythemia was diagnosed in rats whose blood hematocrits (Hct) were $\geq$ 60%. Male (M) and female (F) values are given to the right of total numbers.

TABLE VI. GENOTYPES OF POLYCYTHEMIC LITTERS

| Group [a] | No. of [b] Litters | Litters with Polycythemic rats | | Genotypes [c] (Polycythemic/Total Litters) | | | | |
|---|---|---|---|---|---|---|---|---|
| | | No. | % | AB | AC | BB | BC | CC |
| 1 | 7 | 0 | 0 | - | - | 0/1 | 0/3 | - |
| 2 | 4 | 4 | 100 | 1/1 | - | - | 3/3 | - |
| 3 | 5 | 0 | 0 | - | 0/1 | - | 0/2 | 0/2 |
| 4 | 5 | 3 | 60 | 2/3 | 0/1 | 1/1 | - | - |
| 5 | 4 | 2 | 50 | 1/1 | 1/3 | - | - | - |
| 6 | 4 | 1 | 25 | 1/2 | - | - | 0/2 | - |
| 7 | 6 | 0 | 0 | 0/5 | 0/1 | - | - | - |
| 8 | 2 | 2 | 100 | - | - | - | - | 2/2 |

[a] See Table II for explanation of treatment groups.

[b] No. of litters is the number with pups that survived to weaning at 3 weeks of age.

[c] See Table III for explanation of genotypes. Group 1 rats had 3 additional genotypes not found in any other groups: AE, BD, and BE.

TABLE VII. GENOTYPE AND INCIDENCE OF POLYCYTHEMIA IN INDIVIDUAL LITTERS

| | Litters | | | Incidence of Polycythemia | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | No. Weaned | No. | | % | | |
| Group [a] | Dam [b] No. | | (M F) | (M | F) | (M | F) | Genotype [c] |
| 2 | 6 | A | 0 5 | 0 | 2 | 0 | 40 | BC |
| | 6 | B | 2 1 | 1 | 1 | 50 | 100 | BC |
| | 6 | C | 3 4 | 3 | 1 | 100 | 25 | BC |
| | 13 | C | 5 4 | 2 | 0 | 40 | 0 | AB |
| 4 | 9 | A | 0 4 | 0 | 2 | 0 | 50 | AB |
| | 9 | C | 2 1 | 1 | 0 | 50 | 0 | AC |
| 5 | 13 | B | 3 2 | 3 | 1 | 100 | 50 | AB |
| | 14 | A | 2 4 | 1 | 0 | 50 | 0 | AC |
| 6 | 11 | A | 4 6 | 1 | 1 | 25 | 17 | AB |
| 8 | 17 | A | 7 4 | 0 | 2 | 0 | 50 | CC |
| | 17 | B | 6 4 | 4 | 1 | 67 | 25 | CC |
| TOTALS: | | | 34 39 | 16 | 11 | 47 | 28 | |

[a] See Table II for details on treatment groups.

[b] Dams with the same numerical designation were housed in the same cage until pregnancy when they were transferred to polycarbonate cages.

[c] See Tables III and VI for explanation of genotype symbols.

TABLE VIII. REPRODUCTIVITY OF WISTAR RATS EXPOSED TO METHYLMERCURY, ETHYLUREA, OR SODIUM NITRITE

| Reproductive [a] Parameter | Reproductive Performance [b] for Exposure Groups No. [c] | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 |
| Pregnant dams (No.) | 8 | 13 | 6 | 6 | 6 | 5 | 6 | 2 |
| No. of litters | 7 | 6 | 5 | 6 | 5 | 5 | 6 | 2 |
| Litter rate (%) | 88 | 46 | 83 | 100 | 83 | 100 | 100 | 100 |
| Litters weaned (No.) | 7 | 4 | 5 | 5 | 4 | 4 | 6 | 2 |
| Litter size (No.) | 11.7±1.2 | 10.4±.8 | 12.8±1.0 | 10.7±1.1 | 7.4±1.4 [e] | 8.6±1.0 [d] | 10.7±2.2 | 11.0±1.0 |
| Stillborn pups (%) | 6 | 25 | 7 | 12 | 14 | 21 | 14 | 4 |
| Birth weight (g) | 7.5±.2 | 5.9±.2 [f] | 6.3±.1 [f] | 6.1±.2 [f] | 6.1±.2 [f] | 6.5±.3 [f] | 6.2±.1 [f] | 6.0±0 [f] |
| Pups weaned (No.) | 77 | 24 | 54 | 37 | 36 | 22 | 61 | 21 |
| Survival at weaning (%) | 94 | 39 | 84 | 58 | 84 | 59 | 94 | 95 |

[a] Pregnant dams were detected by examination of vaginal smears and the presence of a vaginal plug. Litter rate is the percentage of pregnant dams that produced litters. Litters weaned is the no. of litters with pups surviving to weaning (3 weeks). Litter size is the average no. of live pups per litter. Percent stillborn pups is the no. dead pups divided by no. of live and dead pups. Birth weight is the average weight of live pups measured by 1 day of age.

[b] Values are expressed as total numbers, percentage, or means ± SEM. Statistical analyses compared control rats in group 1 to the rats from the 7 treatment groups by ANOVA and LS MEANS.

[c] See Table II for exposure details.

[d] $p \leq .10$

[e] $p \leq .05$

[f] $p \leq .01$

TABLE IX. BODY WEIGHTS OF WISTAR RATS EXPOSED TRANSPLACENTALLY TO METHYLMERCURY, ETHYLUREA OR SODIUM NITRITE

| Group [a] | Body weights at three ages [b] | | | | | |
|---|---|---|---|---|---|---|
| | 1 mo | | 2 mo | | 4 mo | |
| | N | WT | N | WT | N | WT |
| | | | MALES | | | |
| 1 | 36 | 41±1 | 34 | 280±7 | 29 | 459±7 |
| 2 | 9 | 41±2 | 9 | 195±2 [e] | 6 | 363±18 [e] |
| 3 | 31 | 38±1 [c] | 31 | 275±8 | 31 | 398±5 [e] |
| 4 | 8 | 37±2 | 8 | 251±9 [c] | 8 | 414±14 [d] |
| 5 | 13 | 42±2 | 11 | 257±7 | 11 | 398±23 [e] |
| 6 | 10 | 44±2 | 9 | 255±9 | 9 | 408±15 [d] |
| 7 | 27 | 36±1 [d] | 27 | 255±3 [d] | 26 | 420±5 [d] |
| 8 | 13 | 37±2 [c] | 13 | 228±5 [e] | 13 | 380±8 [e] |
| | | | FEMALES | | | |
| 1 | 30 | 38±1 | 30 | 203±3 | 32 | 278±4 |
| 2 | 13 | 40±2 | 9 | 179±5 [d] | 8 | 250±9 [d] |
| 3 | 23 | 39±1 | 23 | 214±9 | 23 | 274±4 |
| 4 | 28 | 37±1 | 28 | 185±3 [d] | 20 | 279±8 |
| 5 | 15 | 41±2 | 14 | 178±7 [e] | 13 | 256±8 [d] |
| 6 | 10 | 46±2 [d] | 9 | 192±7 | 9 | 272±10 |
| 7 | 34 | 34±1 [d] | 34 | 197±2 | 34 | 270±3 |
| 8 | 7 | 33±2 | 7 | 166±8 [e] | 7 | 231±17 [e] |

[a] See Table II for details of treatments.

[b] Mean weights (WT)± SEM and statistical analyses were computed with a SAS program. Within columns, values from control rats (group 1) were compared to rats from the 7 treatment gorups. An ANOVA and LS MEANS comparisons were performed to obtain probabilities (P) of significant differences.

[c] $P \leq .05$

[d] $P \leq .01$

[e] $P \leq .001$

TABLE X. WEIGHTS OF POLYCYTHEMIC RATS AND LITTERMATES, AND CONTROL RATS

| Group * | Body Weights [b] at Three Ages ** | | | | | |
|---|---|---|---|---|---|---|
| | 1 month | | 2 months | | 4 months | |
| | N | WT | N | WT | N | WT |
| | | | MALES | | | |
| Controls | 36 | 41±1 [a] | 34 | 280±7 [a] | 29 | 459±7 |
| Polycythemic | 16 | 39±1 [a] | 14 | 208±15 | 11 | 367±10 [a] |
| Littermates | 8 | 43±2 [a] | 8 | 252±10 [a] | 8 | 372±27 [a] |
| | | | FEMALES | | | |
| Controls | 30 | 38±1 [a] | 30 | 203±3 | 32 | 279±4 |
| Polycythemic | 11 | 35±2 [a] | 6 | 153±8 | 5 | 220±21 |
| Littermates | 18 | 41±2 | 18 | 180±4 | 18 | 255±6 |

* Control rats were from Group 1 as explained in Tables II and X. Polycythemic rats from Groups 2, 4, 5, 6, and 8 (see Table V) were compared to non-polycythemic littermates of the same gender.

** Mean weights ± SEM and statistical analyses were computed with a SAS program. Within columns, values with identical superscripts are not significantly different by ANOVA and LS MEANS comparisons at $P \leq .05$.

TABLE XI. HEMOGRAM OF POLYCYTHEMIC RATS COMPARED TO CONTROLS

| Group [a] | N | Hematological Value [b] Hct (%) | RBC ($10^6$/ml) | Hb (g/dl) | WBC ($10^3$/ml) | Platelets ($10^3$/ml) |
|---|---|---|---|---|---|---|
| Controls | 9 | 42 ± 2 | 5.9 ± .3 | 13.6 ± .7 | 10.0 ± .9 | 706 ± 70 |
| Polycythemia | 9 | 67 ± 3 [c] | 8.4 ± .4 [d] | 19.9 ± .9 [c] | 22.9 ± 6.6 [e] | 456 ± 121 [e] |
| Normal | 73 | 39 ± 4 | 6.6 ± .8 | 13.0 ± 1.2 | 8.5 | 330 |

[a] See Tables II and V for details of treatments and polycythemic rat sources. Normal values are taken from pg 1901, Wintrobe, 1983, ref. no. 175.

[b] Values are expressed as means ± SEM as computed with a SAS program, and probabilities (P) of differences between polycythemic and control groups were analyzed by ANOVA and LS MEANS comparisons. Abbreviations are Hct (hematocrit), RBC (red blood cells), Hb (hemoglobin), and WBC (white blood cells).

[c] $P \leq .0001$

[d] $P \leq .001$

[e] $P \leq .10$

TABLE XII. RED CELL MASS OF POLYCYTHEMIC AND CONTROL RATS

| Group [a] | N | Blood Volume (ml) | Red Cell Mass (ml) | Red Cell Mass (ml/100g BW) |
|---|---|---|---|---|
| Controls | 4 | 8.9 ± 1.1 | 3.8 ± .5 | 6.1 ± .2 |
| Polycythemic | 7 | 10.7 ± 1.2 | 6.8 ± .7 [c] | 10.3 ± .5 [d] |

[a] See Tables II and V for details of treatments and sources of polycythemic rats.

[b] Values are expressed as means ± SEM, and probability (P) of differences between groups was analyzed by ANOVA and LS MEANS comparisons.

[c] $P \leq .01$

[d] $P \leq .0002$

TABLE XIII. SERUM ERYTHROPOIETIN LEVELS IN POLYCYTHEMIC AND CONTROL RATS

| Group [a] | N | Hct [b] (%) | Erythropoietin [c] (mU/ml) |
|---|---|---|---|
| Non-Treated Control | 4 | 45±1 | 27±5 |
| Treated Control | 2 | 44±3 | 16±6 |
| Polycythemic | 4 | 63±2 | 968±541 |

[a] Rats were exposed as explained for groups 1 and 2 in Table II.

[b] The mean hematocrit (Hct) ± SEM represents the percent packed erythrocyte volume measured on the day of collection of serum for erythropoietin analysis.

[c] Erythropoietin values were determined with a spleen cell microassay technique by Dr. Krystal, Vancouver, B.C. (ref. no. 89). Values are expressed as mean ± SEM.

TABLE XIV. BONE MARROW AND SPLENIC STEM CELL NUMBERS IN POLYCYTHEMIC AND CONTROL RATS

| Group[a] | N | No. of Cells[b] (x $10^6$) Total | BFU-E | CFU-E | CFU-GM | CFU-M |
|---|---|---|---|---|---|---|
| | | BONE MARROW | | | | |
| Control | 5 | 38±6 | 2.5±.3 | 11.5±1.6 | 17.2±6.1 | 1.3±.1 |
| Polycythemic | 6 | 40±8 | 2.3±.3 | 10.7±1.1 | 6.8±1.1[d] | 1.0±.1[d] |
| | | SPLEEN[c] | | | | |
| Control | 5 | 949±118 | 2.5 | 8.7 | 21.6±7.5 | 9.6±2.2 |
| Polycythemic | 6 | 554±89[d] | 1.5 | 8.5 | 10.8±3.2 | 6.3±1.4[d] |

[a] See Table II and V for details of treatments and source of rats. These rats were all included in the hematology data in Table XII.

[b] Abbreviations for blood stem cells are BFU-E (burst forming unit-erythroid), CFU-E (colony forming unit-erythroid), CFU-GM (colony forming unit-granulocyte/myelocyte), and CFU-M (colony forming unit-monocyte). Values are expressed as means ± SEM and probability (P) of difference between groups was analyzed by ANOVA and LS MEANS comparisons.

[c] Only 1 control and 1 polycythemic sample were tested for no. splenic BFU-E and CFU-E. 5 and 6 samples respectively were analyzed.

[d] $P \leq .01$

TABLE XV. MEAN ORGAN WEIGHTS OF POLYCYTHEMIC AND CONTROL RATS

| Group [a] | N | Body Weight (g) | Percent Body Weight [b] $\bar{x}$ ± SEM | | | |
|---|---|---|---|---|---|---|
| | | | Heart | Spleen | Kidney | Liver |
| Control | 8 | 333±39 | .42±.03 | .22±.02 | .41±.03 | 3.7±.3 |
| Polycythemic | 4 | 126±43 [c] | 1.22±.20 [c] | .55±.08 [c] | .50±.05 | 4.9±.7 |

[a] Non-treated control rats and polycythemic rats were weighed before necropsy, and organs were gently drained of blood and weighed.

[b] Values are expressed as means ($\bar{x}$) ± SEM. Probability (P) of statistical difference between group values was analyzed by ANOVA and LS MEANS comparisons. The P value accounts for any significant age or sex interaction and their effects on the mean weights.

[c] $P \leq .05$

TABLE XVI. CELLULARITY AND MEGAKARYOCYTES IN CROSS-SECTIONS OF FEMURS FROM POLYCYTHEMIC AND CONTROL RATS

| Group [a] | Mean ± SEM [b] | | | |
|---|---|---|---|---|
| | Cellularity | | Megakaryocytes | |
| | N | % | N | No. |
| Control | 2 | 45±3 | 7 | 13.5±1.3 |
| Polycythemia | 4 | 74±5 [c] | 7 | 9.5±1.3 [d] |

[a] See Tables II and V for details on exposure and source of polycythemic rats.

[b] Values are expressed as the mean ± SEM of 4 replicate measurements per rat (N). Cellularity was calculated with computerized stereology to measure the marrow cavity area and cross-sectional area of marrow tissue. Megakaroycytes were point-counted per field at 400x. Probabilities of statistical differences between groups were determined with ANOVA and LS MEANS comparisons.

[c] $P \leq .05$

[d] $P \leq .10$

TABLE XVII. CLINICAL CHEMISTRY VALUES FOR POLYCYTHEMIC AND CONTROL RATS [a]

| Group [b] | N | BUN [c] (mg/dl) | CREAT (mg/dl) | CA (mg/dl) | PHOS (mg/dl) | TOT PROT (g/dl) | ALBU (g/dl) | ALK PHOS (IU/l) | GPT (IU/l) | GLUC (mg/dl) | CHOL (mg/dl) |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Control | 11 | 21.0 ±.5 | .57 ±.03 | 9.0 ±.5 | 7.7 ±.4 | 6.2 ±.1 | 3.18 ±.05 | 197 ±21 | 50 ±7 | 163 ±11 | 51 ±3 |
| Polycythemia | 4 | 24.0 ±3.9 | .55 ±.03 | 8.2 ±.6 | 7.7 ±.5 | 6.3 ±.2 | 2.98 ±.05 | 367 [d] ±25 | 63 ±5 | 118 [d] ±20 | 57 ±6 |

[a] Values are expressed as means ± SEM. Probabilities (P) of statistical differences between groups were determined by ANOVA and LS MEANS comparisons. Abbreviations are BUN (blood urea nitrogen), CREAT (creatinine), CA (calcium), PHOS (phosphorus), TOT PROT (total protein), ALBU (albumin), ALK PHOS (alkaline phosphatase), GPT (serum glutamic pyruvate transferase, or alanine aminotransaminase), GLUC (glucose), and CHOL (cholesterol).

[b] See Tables II and V for details of exposure and sources of polycythemic rats.

[c] Only the test for BUN showed a significant sex interaction with treatment. Polycythemia males had significantly higher levels ($P<.05$) than controls. Polycythemic females had lower BUN levels than control females ($P<.10$).

[d] $P \leq .05$

TABLE XVIII. CLINICAL TOXICITY IN FEMALE WISTAR RATS EXPOSED TO INCREASING DOSES OF METHYLMERCURY

| Methylmercury [a] (ppm in diet) | Dam No. | Degree of Toxicity [b] 6 wk | 7 wk | 8 wk | 9 wk | 10 wk | Reproductivity [c] at 13 weeks (born/weaned) |
|---|---|---|---|---|---|---|---|
| 24 ppm | A | + | ++ | +++ | ++++ | ++++ | 0 |
| 20 ppm | A | + | ++ | ++ | +++ | ++++ | 0 |
| | B | - | + | + | ++ | +++ | 0 |
| 16 ppm | A | - | - | + | ++ | +++ | 0 |
| | B | - | - | - | + | ++ | 0 |
| 12 ppm | A | - | - | - | - | + | 0 |
| | B | - | - | - | - | + | 5/3 |
| 10 ppm | A | - | - | - | - | + | 0 |
| | B | - | - | - | - | + | 0 |
| | C | - | - | - | - | - | 7/7 |
| 8 ppm | A | - | - | - | - | - | 13/4 |
| | B | - | - | - | - | - | 10/8 |
| 4 ppm | A | - | - | - | - | - | 0 |
| | B | - | - | - | - | - | 11/7 |

[a] Methylmercury was prepared as a stock feed of 24 ppm, analyzed for Hg content, and diluted with powdered rat chow to obtain the lower feed levels. Dams were fed the diet for 13 weeks and gavaged during the third trimester (week 13) with ethylnitrosourea precursors as explained in Table II.

[b] The 14 dams on this study were subjectively graded for degree of toxicity as follows: (+) = mild ataxia; (++) = advanced ataxia and circling; (+++) = rear-leg crossover and mild paralysis; and (++++) = total rear paralysis and emaciation.

[c] The rat fed 24 ppm MM died after 12 weeks of exposure. Infertility (inability to cycle or conceive) was common in rats exposed to 10 ppm MM and higher.

TABLE XIX. ANALYSIS OF TISSUES FROM NEWBORN PUPS, ADULT PROGENY, AND DAMS FOR MERCURY CONTENT

| Treatment [a] | N | Levels of mercury [b] (ppm Hg) body | blood | serum | kidney | liver | hair |
|---|---|---|---|---|---|---|---|
| | | 1 DAY-OLD PUPS | | | | | |
| Group 2 | 4 | 19.5 ± 1.5 | | | | | |
| Group 4 | 3 | 16.8 ± 1.8 | | | | | |
| | | 9 MO-OLD PROGENY [c] | | | | | |
| Group 1 | 2 | - | .08 | - | - | - | - |
| Group 2 | 1 | - | 12.3 | - | 36.8 | 17.4 | - |
| Group 4 | 1 | - | 71.5 | - | - | - | - |
| | | DAMS EXPOSED 10 WKS | | | | | |
| 10ppm Methyl Hg | 1 | - | 47.8 | 1.8 | 80 | - | 344 |
| 10ppm Ethyl Hg | 1 | - | 9.6 | - | - | - | - |
| 10ppm Phenyl Hg | 1 | - | .1 | - | - | - | - |
| 20ppm Methyl Hg | 1 | - | 54.5 | 6.2 | 135 | - | 676 |

[a] See Table II for explanation of groups. Note, group 2A was one where dams, after 13 weeks of methylmercury treatment, were replaced on 10ppm methylmercury diets 3 weeks later for about 1 month at which time they gave birth. Ethylnitrosourea precursors were administered during the 3rd trimester.

[b] Levels of elemental mercury were determined for tissues following acid digestion and atomic absorption spectrophotometry.

[c] The group 2 and 4 progeny were littermates of polycythemic rats. See Table VII.

TABLE XX. IMMUNE FUNCTIONS OF WISTAR RATS EXPOSED TO TRANSPACENTAL METHYLMERCURY, ETHYLUREA, AND SODIUM NITRITE

| | | Immune Parameter [b] (x̄ ± SEM) | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | NK Activity | | ELISA | | IL2 | DTH | PGE |
| Group [a] | N | 1:50 | 1:100 | 1:4000 | 1:8000 | (cpm) | (mm) | (pg/ml of supernatant) |
| Control | 8 | 21±2 | 33±3 | 1.19±.11 | .92±.09 | 637±39 | 3.27±.39 | 97±32 |
| Treated | 12 | 21±2 | 32±3 | .90±10 [c] | .68±.07 [d] | 648±52 | 3.30±.26 | 97±27 |

[a] Age, sex and weight matched control rats were compared to rats exposed transplacentally to methylmercury, ethylurea, and sodium nitrite. None of the exposed rats were polycythemic.

[b] See Table IV for details on the one-rat immunoassay schedule and procedures. Natural killer (NK) cell activity is expressed as % cytotoxity per dilution against target tumor cells. Enzyme-linked immunosorbent assay (ELISA) results are expressed as absorbance per dilution at 405 nm. Values are expressed as means ± SEM. Probabilities (P) of statistical differences between group values were determined by ANOVA and LS MEANS comparisons. Age and sex interactions with treatment effects were controlled for in the SAS computer analysis.

[c] $P \leq .05$

[d] $P \leq .10$

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